

Universities learning to play the market

The inability of the British government to match the qualifications of would-be university students to vacancies in science subjects was inevitable given government policies.

THE British government is nothing if not inconsistent. Dedicated as it has been for more than a decade to the principle that market forces will solve most social problems, it is nevertheless "disappointed" at the continuing decline (by a quarter in the past four years) of the numbers of young people leaving school with qualifications of the kind expected by science and engineering departments at British universities. That at least is what Baroness Blatch, the government's spokesman on education in the House of Lords, said last week about this year's crop of statistics of the numbers taking what are called A-level examinations in mathematics and science subjects. But she should not even be surprised. Her government's success in making careers in science and technology less attractive has been amply recorded in the years since 1979. And the screws are still turning; the prospect that basic research will in future be more tightly harnessed to industrial needs will discourage even those who might have been willing to suffer financial indignity for the sake of the traditional intellectual freedom of research. Lady Blatch can be "disappointed" only if she supposes that young people do not read the newspapers.

Of course, poor salaries (especially for graduate students) are only part of the explanation for the continuing unwillingness of young people to compete for places in science and engineering in higher education. The shortage of talented science teachers in schools is another, and it is too soon to know whether the government's present policy of letting secondary schools manage their own budgets if they choose will persuade some of them that paying science teachers more would be prudent. It is just as likely that school governors will argue that because the 'demand' in schools for A-level science is falling, they had better divert resources in other directions. Is not that the logic of the market?

Among the other partial explanations of the decline of science numbers is an archaic feature of the British educational system that Lady Blatch described last week as the "cornerstone" of her government's plan to promote "higher standards of achievement among young people". The reference is to the system of A-level examinations that has become the *de facto* entrance examination into higher education. And nowhere can it be denied that the system is a remarkable venture in cultivated precocity. Young people leaving British secondary schools with decent A-level grades probably know as much about their chosen subjects of study as do undergraduates elsewhere after their first year in higher education. What suffers is general education,

illustrated by the notorious inability of British science school-leavers to speak any language but English (and, sometimes, not even that).

This state of affairs will not be remedied by the government, wedded as it is to the belief that skewed A-level attainment is its proudest achievement. Is there a chance that some among the universities (of which Britain now has many more, of great diversity) will have the wit to find a way out? After all, the ingredients of a better system are all in place. For one thing, the A-level pattern has recently been made less rigid by allowing students to follow broader courses (but very few choose to do so for fear of not commending themselves to their chosen universities). For another, the universities are themselves being forced towards charging fees for tuition by the government's attempt to rig the educational market in favour of science, implied in its reduction of the annual per capita payment to universities in respect of students in other fields. Canny universities will quickly tumble to the conclusion that they will be better off financially if they can persuade students in the arts and humanities to switch to science. It will be a short step from there to the conclusion that they might just as well short-circuit the A-level system altogether, choosing students by other criteria. And if that is the likely outcome, why not anticipate it? That is what the logic of the market commands. □

Babbitt on a roll

Bruce Babbitt, has had a greater effect on the environment in six months than his predecessors had in years.

In the US hierarchy of government posts, the secretaryship of the Department of the Interior has never reached the pinnacle. In fact, the names of most interior secretaries are forgotten the day after they are sworn in. Not so Bruce Babbitt, a man with a clear (and sensible) vision of the environment as a complex, live phenomenon and a man who has the president's ear.

Within weeks of taking office, Babbitt took steps to end an endless stalemate between environmentalists who wanted to protect every living red cockaded woodpecker and lumberers who wanted to cut down trees where the endangered birds live. Babbitt forged an agreement that permits lumbering provided companies preserve at least 10 acres of



trees standing around each colony of woodpeckers living on their land.

The new secretary also announced a plan to end long-standing turf wars among various interior department agencies by bringing staff scientists together under the umbrella of a National Biological Survey (similar to the US Geological Survey founded a century ago). The survey, which is likely to become reality this fall if, as expected, Congress passes the necessary funds (see page 751), is designed to separate science from research management so that "we can elevate the credibility of science itself", Babbitt has said.

These are both good moves. But Babbitt showed his mettle (and President Bill Clinton's capacity for deviousness) most demonstrably two weeks ago when he announced higher fees for ranchers who graze their herds on public land (see *Nature* 364, 659; 1993). Although the increase from \$1.86 a month per 'animal unit' to \$4.28 a month is likely to be reduced as the Babbitt plan wends its way through a public rule-making process, its symbolic importance cannot be overstated. An increase in grazing fees was originally in Clinton's economic package, the first of many sound measures the president traded for key congressional votes. But within days of the passage of the fee-free package, the idea that ranchers should pay closer to the market rate for US rangeland forcefully reared its head in the form of Babbitt's proposal, which does not require the blessing of the Senate or the House of Representatives.

This week in Alaska, Babbitt helped end a three-day protest by promising fishermen more help in recovering from the 1989 *Exxon Valdez* oil spill. The fishermen, who had blocked 60 oil tankers from reaching the Trans-Alaskan pipeline, attribute the recent death of salmon and herring to the spill, which dumped nearly 11 million gallons of crude oil into fishing waters. Babbitt ended the protest by promising to urge Exxon officials to negotiate settlements with the fishermen and to promote aid and protection for fish hatcheries and spawning streams.

Next on the Interior Secretary's agenda are wetlands policy and higher user fees on mining, timber and water rights to federal lands. His purpose is not to put ranchers or miners out of work but rather is to strike a balance between their needs and the need to preserve ecosystems by requiring users to take new (and more costly) steps to preserve the public lands from which they profit. Babbitt has the right ideas. □

Pfizer donates drugs

A pharmaceutical company's public relations policy is also good for public health

IN a move aimed, no doubt, at the Clinton Administration, which has made US pharmaceutical companies out to be the villains in its health care reform plan, Pfizer Inc. of New York has struck a powerful blow. Hillary Rodham Clinton, in speeches throughout the United States, has attacked the cost of drugs as one of the principal factors driving the US health

care bill into the stratosphere. Not surprisingly, the nation's large pharmaceutical companies, which are not only profitable but also highly successful in the international market, argue that the Mrs Clinton is wrong — that the price of drugs reflects the high costs of research and development, not greed.

Now Pfizer has decided to add another arrow to the industry's response to the Clintons. The company has announced that through a new programme charmingly called "Sharing the Care" it will donate to the poor and homeless all 11 of the drugs it makes that are not reproduced by other companies. "Through community, migrant, and homeless health centres in all 50 states [Pfizer] will provide single-source pharmaceuticals at no charge to patients who live at or below the poverty line", says a company spokesman.

Among the free Pfizer drugs are some of the most medically important: Procardia XL, a calcium channel blocker for the treatment of hypertension, which is widespread among the poor; Diflucan, an anti-fungal agent useful in the treatment of patients whose immune systems have been compromised by AIDS, cancer or organ transplantation; and Glucotrol, a once-a-day drug to lower glucose in diabetics — another widespread problem among the population Pfizer is promising to help.

"Sharing the Care" is an expansion of charitable programmes already run by Pfizer and other drug companies that donate certain drugs to health centres for the poor. From a public relations point of view, it is an expansion likely to meet with approval from the Clintons' health care advisers, but it is unlikely to change their overall view of the pharmaceutical industry as a force that drives up costs. But, beyond that, it is sure to strike a positive chord with physicians and nurses ministering to those in desperate need of these drugs. In this case, what is good for Pfizer is also good for medicine. □

Only in California

A school board in the town of Vista, California, has ordered the teaching of creationism in public schools.

The teachers are against it. So are many parents. And scientists in California are duly offended by a local decision that violates state education policy. Nonetheless, by a vote of 3:2, members of the 'Christian Right' in Vista voted that the school curriculum should include the teaching of creationism in history, social science, literature or science courses "at appropriate times". That apparently means any time that mention is made of divine creation or the ultimate source or purpose of life on this planet.

The temptation to rail against creationism, marshalling all of Darwin and the scientific literature of palaeobiology in defence of evolution, is strong but most likely futile. Some matters are not susceptible to resolution by reasoned argument and this (poor Darwin) is one of them. There will be lawsuits to overturn the Vista creationists, based on arguments that they are illegally promoting a single religion. Who knows, the good guys may win. But at least one can say it happened only in California. □

Mississippi subsides, leaving research debate in its wake

Washington. As the great Mississippi floods subside, hydrologists are looking to greater research and stronger models to better manage the nation's flood plains.

Conservation groups have attacked the Clinton Administration for failing to coordinate the various agencies that are looking at what now should be done. The 15,000-strong American Rivers group, which is worried that flood control levees on the

just the first instalment. Meanwhile, reports by eminent persons on how to manage river basins better have been gathering dust.

"You've got to take the opportunity to change the system", says Eugene Stakhiv, chief of policy at the Corps of Engineers' Institute of Water Resources at Fort Belvoir, Virginia. The Corps has had a long and frantic summer. It built the physical flood control barriers on the upper Mississippi, and has been subject to considerable criticism, much of it ill-informed. It is keeping an open mind on how to respond to the floods, according to Stakhiv.

Stakhiv, who attended Ragone's panel meeting, says the main conflict is between scientists who want to gather data to build reliable hydrological models in the longer term, and those who want data to make decisions on such urgent matters as whether to reconstruct levees on the Mississippi.

What is most important is that the various agencies should agree upon a strategy. "Let's assume we get \$100

million research money out of the flood", says Stakhiv. "Unless we get the agencies to agree on a programme, it'll be dispersed and there will be no integration. That is what all the various task forces are trying to deal with."

Flood management veterans say that the government has already been given advice on how to achieve such coordination, but has not taken it. Gilbert White, founder of the natural hazards research centre at the University of Colorado, says: "This gives the US the chance to take a fresh and bold look at what it's been doing. Instead of flood control we should be concerned with the wise use of flood plains, which may involve flood control." White wants the administration to implement the unheeded recommendation of a report, produced last year by an interagency task force, that the federal government should define good flood plain management, and set goals and a timetable for its implementation across the country.

In his drive for 'smaller' government, President Ronald Reagan disbanded the US Water Resources Council in 1982. Follow-

ing the Mississippi floods, Clinton will probably restore some form of national coordination.

But the important decisions on the best response to the flood will be taken at a lower level, out of the public eye, and will be somewhat more sophisticated than the public battle between the Corps of Engineers and the green lobby would suggest.

Kevin Coyle of American Rivers sums up the green case when he says that "the whole idea of river engineering is flawed". But Coyle was stopped in his tracks when a journalist pointed out that 40 deaths, however regrettable, was a very small number for such an angry Mississippi to extract in a heavily populated area. "Without the levees, this disaster would have been worse", says Stakhiv. "The levees gave people time. Nothing is perfect: all we can do is mitigate the worst of the hazards."

Engineers and scientists will now work to mitigate them further, with more support than they have had in the past and perhaps within an integrated framework decided by the federal government. "The effect of the floods is to draw attention to the situation", says White. "The precedent is that major policy changes in this country often come from an emergency."

Colin Macilwain

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Radar images from the ERS-1 satellite, shown purple-blue over flooded terrain, reveal the extent of flooding near the Mississippi-Missouri-Illinois confluence, 2 August 1993.

Mississippi will simply be rebuilt, has called on Clinton to set up a panel to review not only flood control on the Mississippi but also national flood control policies. "There is no-one in charge of US flood control", complains its director, Kevin Coyle. "Nothing is happening," says Beth Norcross, legislative director of American Rivers. "It is sitting in the White House's lap."

But scientists from the various agencies are already coordinating their activities both officially and informally. The Federal Coordinating Council for Science, Engineering and Technology has set up several committees to coordinate a scientific response, following a request from Jack Gibbons, head of the White House Office of Science and Technology Policy. One committee, under the chairmanship of Stephen Ragone of the US Geological Survey, met last week to discuss research priorities.

The situation is a classic example of how science can serve — or fail to serve — government. Clinton recently went to St Louis, Missouri, to present \$7 billion of emergency aid to the populace, and that is

Community values

Munich. The European Commission is to organize a "week of science culture" in November to raise the profile of European science. The research commissioner, Antonio Ruberti, brought the idea with him from Italy where he was minister for research until last year. He introduced the annual Italian week of scientific culture in 1991.

The twenty-odd events will range from competitions to conferences and will be widely publicized. Participants are not confined to EC countries: several other West European countries are taking part. Organization of each national project will be shared with other European countries.

Most of the large international European laboratories are also taking part. The European Southern Observatory (ESO) at Garching in Germany, for example, will hold an essay competition on astronomy for high-school students in 17 European countries and also in Chile where it has a large observatory. The 18 winners will spend two weeks with ESO scientists working on genuine research projects, which will eventually be published. They will also spend three nights in Chile using ESO's most powerful telescopes.

Allison Abbott

Fight for resources spells trouble for Polish institutes

London. Poland's research institutes are for the first time competing for a slice of the research budget, which has shrunk by two-thirds over the past five years to 19,000 billion zloty (a little over US\$1 million at today's exchange rate).



Jan Krzysztof Frąckowiak: feeling positive about prospects for Polish science.

One in eight institutes has been cut off from state funding, and one in four has seen support reduced to well below basic running costs, following the introduction of a policy to allocate more funds to the best-performing institutes: under communism the state guaranteed the running costs of every institute.

The new policy allocates almost two-thirds of all funding to institutes rated 'A' (international level) and one fifth to those rated 'B' (high national level). Institutes rated 'C' (need to improve) receive little money, while 'D' spells disaster — no funding.

To decide who should receive what, Poland's new committee for science research (KBN, see sidebar) ranked more than 850

university departments and research institutes according to publications, citations, and graduate output.

Almost a third of Poland's institutes fell into the bottom two categories. To avoid extinction, many have begun selling services to industry, with conspicuous success: last year they earned much of the total industrial income of Poland's institutes (Z13,000 billion — more than a third of the research budget). But nobody knows how many institutes went to the wall.

Some complain that the assessment — which they otherwise endorse — shows that the move away from communist influences is disappointingly slow: almost all the 83 institutes that previously belonged to the Polish Academy of Sciences appeared in the top two categories.

But Jan Krzysztof Frąckowiak, a former academy physicist and now secretary of the KBN, argues that the institutes were always renowned for high quality research. They were often used to house "politically incorrect" researchers, he says, who were unable to follow normal university careers.

KBN has also introduced greater competition for funding of research grants. In 1991, it began awarding grants according to a peer-review process that ignores the applicant's career status and the rating of the institute where he or she works. Under communism, a young postdoctoral fellow could rarely compete for funds with

KBN bringing democracy to Polish science

Western practices of science administration came to Poland in 1991 — two years after non-communists came to power in the first democratic elections — when the government created a state committee for science research (KBN) to take over from the communist-controlled Polish Academy of Sciences. The committee has introduced a more selective system for distributing the research budget and created formal evaluation procedures.

The democratic constitution of KBN is a matter of great pride. Besides five government representatives, the committee comprises 14 scientists elected by the scientific community. KBN's two scientific commissions for basic and for applied research are also elected. They advise the committee where to spend research money using new performance-based criteria. **B.I.**

an established professor.

The major problem now facing KBN is that it does not have enough money to support fully even highly rated institutes. On average it has paid institutes almost a third less than they asked for, and has funded only a quarter of the 28,000 grant applications; good applications are sometimes held over to the next round (there have been five so far) to give them a second chance.

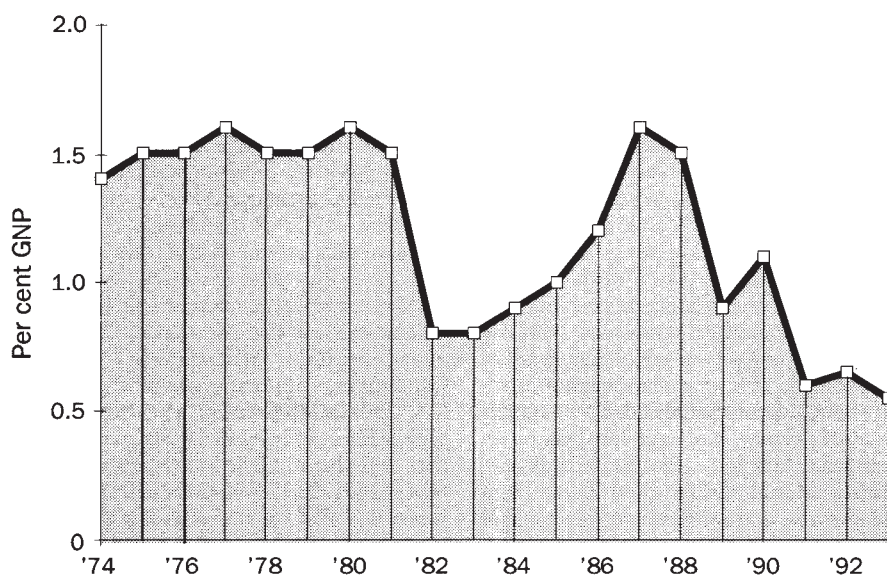
Science spending has dropped from a peak of 1.5 per cent of gross national product under communism, to less than 0.6 per cent this year; this compares with an average of around 2 per cent in Western Europe. Inflation has further eroded the buying power of this year's research budget of Z18,970 billion.

The shrinking budgets have also triggered an exodus from science: almost one-fifth of the researchers in Poland left science last year. The uncertain prospects also mean that talented students no longer view a research career as attractive.

Scientists attitudes are also slowing reform. Too many older and established researchers find it "degrading" to have to ask for money, says Maciej Żylicz, a molecular biologist who is prorector for science at the University of Gdansk.

Nevertheless, KBN's members are optimistic. Poland is again between governments, following the defeat of Hanna Suchocka's coalition in May, and will hold a general election in mid-September. But KBN has already outlived four governments and is confident that political instability will not threaten its future. **Barbara Izdebska**

Spending on science in Poland (per cent GNP) 1974 – 1993



Source: Rzeczpospolita

Soros money filters through

Moscow. Scientists in the former Soviet Union (FSU) this month began to collect survival cheques from the \$100 million-fund set up last year by the US philanthropist George Soros. But doubts persist about whether the next and main round of research grants will be as successful in finding and funding the right scientists. Many are also sceptical about whether the money will make any long-lasting difference.

In this first phase, Soros's International Science Foundation (ISF, see *Nature* 360, 617; 1992) is distributing grants of \$500 to up to 20,000 individuals who have published at least three papers in recognized journals over the past five years (see *Nature* 362, 95; 1993). Applicants with good citation records receive a further payment of up to \$1,000 to help keep their research team intact.

ISF has overcome early concerns that it might be impossible to get the money to scientists, given that the FSU lacks a reliable banking system, and the alternative of wiring money directly to researchers was too expensive. It has paid researchers in US traveller's cheques, which they convert into cash at Moscow's Menatep commercial bank.

Officials at ISF's office in Moscow are also confident that the money went to the right people. But statisticians may raise an eyebrow at their survey method: "we became convinced of this by looking at the people who came to pick up the cheques."

The rescue plan will now move to its second and main phase of handing out research grants. But controversy still surrounds how best to spend the \$100 million pledged by Soros, says Alex Goldfarb, who supervises ISF's Moscow office. Soros and many Russians believe it should be shared among the 25,000 or so researchers active in basic research. Others, particularly the US members on ISF's executive committee and advisory board, argue it should be used to ensure the survival of the best research groups. Unfortunately, \$100 million is not enough to do both.

ISF has reached a compromise for the next round. Besides awarding grants, of up to \$100,000, to support large research projects, it will also make available some smaller grants to help research groups in danger of disappearing.

But Russia's top scientists may prefer to emigrate than face the "humiliation" of accepting the \$350 maximum monthly salary on offer, warns Maxim Frank-Kamenetskii of the Institute of Molecular Genetics in Moscow: "It would have been acceptable a year ago," he says, "but the purchasing power of the dollar is plummeting, and prices of basic goods are approaching those on the world market. Such terms deprive serious scientists of their last hope of working productively in their own country."

Getting grant applications peer-reviewed

by Western scientists is another problem facing the second round. Unlike large research foundations in the West, ISF does not have the resources to maintain an active network of referees. It intends to send applications to potential referees without notice, and is concerned that few may reply.

But the major question is whether Soros's \$100 million can make any permanent difference given the difficult economic situation in the FSU. It needs to run for five years, not one, if it is really to help, says Goldfarb: "We have saved a person from drowning by the hair, and are holding his head above water. But we will soon drop him, and swim ashore empty-handed." Soros's call for others to join the ISF programme has gone unheeded.

Vladimir Pokrovsky

Israelis propose building tokamak in Russia

Jerusalem. Israel is studying a proposal to order from Russia a small-aspect ratio tokamak as part of a joint fusion research programme.

Michael Finkenthal of Hebrew University and Dr Moshe Rosenblum of the Israeli Atomic Energy Commission presented the plan to Israel's new energy minister, Moshe Shalal, earlier this month. The Ministry of Energy is "actively considering" the proposal, says its chief scientist Amnon Einav.

The scientists advise against buying an existing Russian tokamak as proposed by Russia earlier this year. Instead, they recommend that Israel adapt the small-aspect ratio tokamak design developed at the Yoffe Institute in St Petersburg. They say it has advantages over the conventional tokamak and could be adapted for research into the important area of steady-state operation. Under the joint programme, a similar device would be built for Russian researchers. Constructing a small tokamak in Russia could cut construction costs to US\$5–\$10 million, say Israeli scientists.

E. P. Velikhov of the Russian Academy of Science proposed the joint venture to the Israeli prime minister, Yitzhak Rabin, earlier this year. He suggested it could give Israel a stepping-stone to the International Thermonuclear Reactor Programme: Russia takes part in the programme — along with the United States, Europe and Japan — and can invite other countries to join its efforts.

Prospects for a national fusion programme in Israel have been improved by the arrival of immigrant fusion scientists from the former Soviet Union. "Before, we lacked a critical mass of scientists with hands-on experience" says Finkenthal. Eleven such immigrants have now been told that they may yet find work in their field — something most of them had given up on.

Abraham Rabinovich

Lab practice helps poor kids

Washington. A long-running scheme that lets US high-school students from poor families work in research laboratories during the summer has been an overwhelming success, the American Chemical Society (ACS) annual meeting in Chicago was told this week.



Theodore Goodson

More than three-quarters of the students surveyed in the city of Indianapolis who participated in the ACS's Summer Educational Experience for the Disadvantaged (SEED) scheme went on to obtain college degrees, according to Ted Goodson, a research manager at Eli Lilly.

Goodson's retrospective study of the scheme's track record over 20 years in Indianapolis "proves the sceptics wrong", he says. A symposium and luncheon at the Chicago meeting will celebrate 25 years of the scheme's operation during which around 4,000 teenagers from most states have spent

8 to 10 weeks working as research assistants.

When ACS launched the scheme after the riots that followed the assassination of Martin Luther King in 1968, few believed it would succeed, says Goodson: "School teachers were reluctant. They thought the laboratories would be too sophisticated an environment for the kids. The researchers were high-minded, as you would expect, and the corporations thought the scheme was too optimistic." But they now enthusiastically support it, he says.

But nationally, where ACS administers the programme while seeking external support to pay the stipends of \$1,200 per student, the scheme is struggling to attract enough money to maintain last year's peak throughput of 300 students.

And if SEED's founders hoped to adjust the social and racial balance of American chemistry, a visit to Chicago will merely confirm that US science falls far short of "looking like America", to use President Clinton's celebrated phrase. "There's a long, long way to go", concedes Christine Brennan of ACS. "But we've helped a lot of students to gain the confidence to continue education beyond high school."

Colin MacIlwain

Gene bill still controversial

Munich. Austrian academics and industrialists have welcomed a revision of the draft gene bill that would simplify safety requirements and reduce the administrative burden on researchers. But controversy continues because the bill would legislate not only on safety but also on social and ethical aspects of applications such as gene diagnosis and therapy. Critics say existing laws should be amended to cover such areas.

Austria's Socialist health minister, Michael Ausserwinkler, revised the bill following protests that it was 'hyperbureaucratic and antiresearch'. Scientists argued that the approval procedure would delay experiments as it used too many unscientific criteria. Other interest groups attacked the bill as scientifically and ethically confused.

Erhard Busek, the science minister, argued that the failure of Germany's gene law (see *Nature* 363, 481; 1993), on which Austria modelled its bill, showed that such restrictive legislation is unworkable: Ausserwinkler needs Busek's support because the cabinet must approve the bill unanimously.

The bill's major concession is that researchers would not have to seek case-by-case authorization for experiments falling into the lowest of the four designated safety grades: 'low or no risk to humans and the environment': they need only be registered. Higher risk experiments would be authorized within 90 days. A national committee

for biological safety and the health ministry's scientific committee on genetic engineering would assess safety levels. In Germany, such low-risk experiments still require individual authorization, although this is being hotly debated in parliament.

Safety requirements for the release of transgenic plants into the environment remain similar to those in other countries: field trials would need to be carried out in stages, each approved by a safety commission. Procedures will, however, probably be simplified for experiments already carried out elsewhere or involving release of plants considered as "easy to recover". The revised bill drops the question of liability in the event of environmental damage.

Still controversial, however, is Ausserwinkler's plan to include in the bill provisions on social and ethical aspects of gene diagnosis and therapy. Politicians across Franz Vranitzky's coalition government disagree over whether such a comprehensive gene law makes sense.

Some argue that existing laws cover many applications of genetic engineering, particularly in medicine. They believe it would be better simply to amend such laws to take new techniques into account, as most European countries have done. Legislating for such things as gene therapy within a safety law, they claim, could hinder progress by implying a level of danger that does not exist.

The bill would ban germ-line gene

therapy. It would allow somatic gene therapy for serious hereditary diseases and cancer but not for cosmetic purposes, and create a ministerial committee — along the lines of the US National Institutes of Health's Recombinant Advisory Committee — to authorize applications. A local ethics commission at each clinic would approve protocols.

Amendment of existing drug laws would make more sense, says Nikolaus Zacherl, administrative director of the Institute for Molecular Pathology in Vienna: "The health ministry is only interested in making a name for itself and in assuming a pioneering role in the world with this new gene law."

The revised bill relaxes a requirement that patients should approve any medical analysis using genetic techniques. This would have put the onus for technical decisions on non-experts. Patients would still need to consent to genetic analyses used to diagnose hereditary diseases or to identify genetic predispositions. But routine methods for monitoring therapy, such as HLA cell-typing during leukaemia treatment, would be exempted.

Patients diagnosed with genetic disorders would also have to attend genetic consultations. Christine Mannhalter, director of the department of molecular genetics at Vienna's General Hospital, complains that it is difficult to imagine who would provide the service because Austria has no training programme in human genetics.

Other parts of the bill would forbid employers and insurance companies to use genetic data to screen applicants.

Robert Unterhuber

Earthlings interfere in extraterrestrial search

Santa Cruz, California. Electromagnetic radiation from Earth is interfering with the controversial \$104-million hunt by the US National Aeronautics and Space Administration (NASA) for little green men.

NASA started its ten-year Search for Extraterrestrial Intelligence programme (SETI) last October, after years of wrangling with legislators who called it a waste

of taxpayers' money. At least eight radiotelescopes in the United States and Australia are expected to take part, scanning millions of radio frequencies for signs of intelligent life. Some will home in on stars most likely to have habitable planets (Targeted Search). Others will sweep the heavens indiscriminately (Sky Survey).

Last autumn the Targeted Search team, led by Jill Tarter, logged more than two-hundred hours of observations of 25 stars using the Arecibo Observatory in Puerto Rico. They found the incidence of false alarms, caused by radio interference, to be much higher than expected. NASA will take steps next year to avoid such a waste of scarce and expensive observation time. It will equip each radiotelescope with a follow-up device, which would continue to analyse a puzzling signal at higher resolution; meanwhile, the rest of the equipment would scan the same star at other frequencies.

The agency will also begin observing SETI signals interferometrically, by combining signals from the same star detected simultaneously by observatories far apart. Software would eliminate interference from

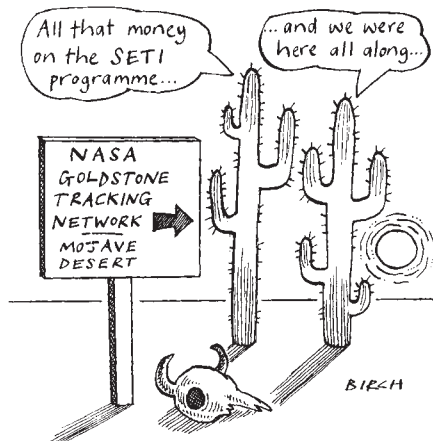
Earth, identified as signals that do not stay fixed with respect to the stars as the Earth rotates.

The Sky Survey part of SETI has not encountered any more false alarms than it expected. It operates from the 34-metre radiotelescope at NASA's Goldstone tracking network in the Mojave Desert in California, and the frequencies it scans are free of interference.

News of the unexpected interference in the Targeted Search programme was announced at last week's International Astronomical Union conference on the search for extraterrestrial life.

At last year's meeting, scientists from the former Soviet Union were enthusiastic about conducting joint SETI searches with NASA. This year, only three Russian scientists, all on sabbatical in the United States, took part; two invited researchers did not turn up. "The United States is rich enough to afford SETI," explains Vladimir S. Strelitski, a former vice-president of a Russian SETI commission, who now works at the National Air and Space Museum in Washington, "Russia isn't."

Keay Davidson



MicroGeneSys goes on trial

Washington. The US Army is to carry out the world's largest trial of a therapeutic AIDS vaccine in the face of fierce opposition from many AIDS researchers, after agreeing supply of the vaccine from the Connecticut company, MicroGeneSys.

The Army and MicroGeneSys appear to have outwitted AIDS activists and scientists who thought they had blocked the controversial single-product trial last April, when, as a result of White House intervention, responsibility for the trial was transferred from the Army to the National Institutes of Health (NIH).

But NIH's plans for a comparative trial of several therapeutic vaccines had to be abandoned in June, when MicroGeneSys refused to provide its vaccine free (see *Nature* 363, 294, 1993). The Army trial will now test only the MicroGeneSys vaccine, which will be paid for by its commercial partner, Philadelphia-based Wyeth-Ayerst.

Final details of the trial have still to be negotiated with the Food and Drug Administration. But 5,000–10,000 HIV-infected patients will probably be injected with either vaccine or placebo over four years. The Army will assess the outcome.

Frank Volnovitz, president of MicroGeneSys says the agreement with the army is "fortunate", and that the company's

vaccine is "the only one ready to enter Phase III trials". He dismisses NIH's opposition to the Army's trial as "really about protecting turf, not to do with AIDS": a high-level NIH panel has unanimously agreed that there are no scientific grounds for the trial.

Volnovitz denies that recent events have undermined the scientific basis of the planned trial (see *Nature* 364, 374; 1993). But he concedes that "there is a prevailing opinion [among senior AIDS researchers] that this is the case". He says that the trial's basis has been "no more undermined than the use of surrogate markers". The huge trial will look to patient survival as its main marker, and not CD4 counts.

Wyeth-Ayerst will pay MicroGeneSys an undisclosed sum, believed to be between \$5 million and \$10 million, to cover the cost of producing vaccine for the trial. A spokesperson for Wyeth-Ayerst says that the company did not offer to pay for the vaccine when NIH wanted to use it in the multiple-product trial because "NIH never asked us". Opponents of the single-vaccine trial take comfort from the fact that Wyeth and not the taxpayer is paying this sum. But the fact remains that the Army and MicroGeneSys have both got almost exactly what they wanted.

The trial will probably start in the new

year, even if Congress decides to investigate how the \$20 million cost of the trial became earmarked to the Army last autumn. Volnovitz says he would "welcome as much investigation as possible into the whole area".

Tony Fauci, director of the National Institute of Allergies and Infectious Diseases and the chief AIDS researcher at NIH, declined to comment on the outcome: "We gave our recommendations, and have nothing more to add." **Colin Macilwain**

New agency pools biology research

Washington. Biological research scattered throughout the US Department of the Interior will in October be brought under the umbrella of a new agency, the National Biological Survey (NBS). One task of the agency will be to compile an inventory of every ecosystem in the United States and its plant and animal species.

NBS will regroup the Department of the Interior's budget for biological research with an expected small increase to \$164 million; Congress is expected to approve the NBS budget next month. Similarly its staff will be drawn from a reorganization of the 1,700 scientists in its various laboratories. The new structure should use resources better, by reducing fragmentation and duplication, and provide a clearer long-term strategy.

"What we're aiming for is good science, science that is unbiased and solid enough to bring everyone together at the negotiating table talking the same language", says NBS spokeswoman Trudy Harlow. One way in which independence and objectivity will be encouraged is by separating scientists from decision-making and enforcement.

Bruce Babbitt, Secretary of the Interior, sees good science as the means to streamline ecosystem management and to avoid "environmental train wrecks". He says a recent court dispute — between environmentalists and the timber industry over the spotted owl in the Pacific Northwest — would not have happened if the department had collected more long-term scientific data on such things as population counts.

But critics say more information is not enough. "NBS will only generate more paperwork in Washington. It's purely cosmetic", says Jerry Taylor of the CATO Institute, a conservative Washington think-tank: "The only way to prevent extinction is to provide economic incentives to landowners, to make it in their best interest to comply with species protection laws."

Even environmentalists, who applaud the creation of the NBS, agree. "All the databases in the world won't help until we complement the Endangered Species Act with more comprehensive initiatives to maintain the natural habitat", says Mark Shaffer of the Wilderness Society. **Susan Greene**

Supercollider tunnelling stops

Dallas. The last of the tunnelling machines boring the main, 54-mile circumference ring to house the Superconducting Super Collider (SSC) will grind to a halt next week, to await Congress's final verdict on whether the \$9-billion project can proceed.

Two machines have stopped and the two others are proceeding to positions where they too can safely come to a halt, probably on 30 August and 4 September respectively. In common with other construction contracts, the tunnelling is being suspended as cash dries up and the SSC conserves what money it has to pay its 1,700 core staff.

The project director, Roy Schwitters, says he remains in the dark about the plans of Energy Secretary Hazel O'Leary to appoint a new lead contractor to manage project construction (see *Nature* 364, 567; 1993). "I don't know what model they are considering", he says. But he hopes that the changes will leave most of the existing project management intact, including his own position. "I don't anticipate leaving the project", he says. "I'm interested in seeing the SSC built, and I'll do anything to further that goal, whether that means staying or not staying."

Opinion is divided at the SSC's bustling design laboratories in the suburbs of Dallas, and on the sprawling construction sites

around Waxahachie, just south of the city, as to whether the project will survive. Scientists are proceeding on the basis that the project will survive, but some local people are under the (mistaken) impression that Washington has already decided to kill it.

For his part, Schwitters considers it "likely" that the SSC will survive. "I think there are a lot of serious people in Congress who are beginning to fathom the consequences of stopping this", he says. The House of Representatives has voted not to fund the project (see *Nature* 363, 6; 1993) but the Senate will probably support it next month, leaving the final decision in the hands of an unpredictable conference between leaders of the two houses.

Schwitters warns that uncertainty over the project's future has prevented the management from developing plans to stretch out the project, as it was asked to do by the Clinton administration in the spring. "That work is not where it should be", he says, "so there is going to be a multi-month effort to re-invent the project" if funding is indeed resumed. "What the impact is on the final completion date I can't tell you. I sense we could maintain the 2002 date suggested by Clinton; the [original] 1999 date is very hard to imagine." **Colin Macilwain**

Maastricht Treaty undemocratic

SIR — Your leading article “Can Europe ever be made to function?” (*Nature* 364, 467; 1993) states that “[the] chief goal [of the Maastricht Treaty] is . . . to replace all 12 European currencies by a single European currency”. Chief goal? The “Treaty on European Union” contains seven ‘titles’, six of them of substantive importance. Among many purposes, only one could be called “chief”: that declared by the treaty’s name, and Title I, the establishment of a (political) European Union. This is a vastly larger project than a single currency, although monetary union would no doubt so tie the hands of national governments that any pretence of national independence would rapidly be seen to be just that. Currency union, the subject of only parts of Title II of the treaty, is thus a means, but not the end.

The treaty declares as a fundamental constitutional principle the agglomeration of political power into the hands of European Community (EC) institutions (the *acquis communautaire* of Article B — it is surely significant that no English translation of this phrase has been found). It is also clear that the executive and legislative apparatus exercising that power will remain on a nondemocratic basis (notwithstanding the byzantine, and merely incremental, reforms of decision-making in Articles 189 (a–d) (Treaty of Rome as amended under Article G of Maastricht)). European citizens thus face surrender both of national sovereignties and also, more importantly, surrender of parliamentary democracy.

You excuse the failure of the timetable for monetary union as a project that got too far ahead of “general cohesion”, and you describe both as “excellent objectives”. But why should one find the objectives excellent? The treaty is a fundamental constitutional document in which democracy has been almost forgotten. You call for EC institutions to be placed on a “more secure and rational foundation”; yet they are already all too secure, and, for the purpose of dragging the continent into a nondemocratic super-state, all too rational. The fundamental flaws of all the European treaties and institutions, unacceptable to millions, but which you fail to identify, are the absences of (1) a thorough-going democratic constitution, and (2) clearly drawn limits to EC jurisdiction.

To those of us who still believe in the vote and representative democracy as the only satisfactory basis yet found for civilized government, the recent setbacks for monetary union will be seen as a glimmer of hope; there is now some prospect that the glue intended to bind unwilling subjects to the political objectives of the treaty will come permanently unstuck.

From such a viewpoint, monetary union will not be seen in a different light until democracy replaces bureaucracy and technocracy as the executive and legislative basis of the EC. That, however, requires standing the Maastricht Treaty completely on its head.

To *Nature* readers from the United States, whose founding fathers (unlike those of the EC) cared deeply about democracy, your sentiments might appear, in the light of the actual treaty text, distinctly peculiar. To readers from newly democratic European countries, they ought also to be troubling: the democratic criteria for admission should not delude them into supposing that the EC will itself be democratic. It is significant that (in the United Kingdom at any rate) only bitter opponents of the treaty encouraged us to read it. I began this letter supposing that your leader-writer had not; some proponents in the government even admitted to this. Reading it, belatedly, was an eye-opener for me; I urge readers in any country currently considering EC membership to do the same, as a vital duty to themselves and their countrymen.

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Not so free trade

SIR — Europe is now without frontiers. There is free passage of goods and people across national borders. As this is the case, I would like to ask the manufacturers of scientific equipment and consumables why I have to pay a premium of more than 100 per cent on some goods for working in Italy? A number of manufacturers refuse to supply goods across national boundaries, referring me back to the local distributor. In one case, the local distributor charged a sterling equivalent of £370 when the price in England was £165. Furthermore, the local distributor did not want to send my order to the parent company in the United States until he had received a few other orders, necessitating a wait of well over a week if the required additional orders did materialize, and well over two if they did not. The English supplier had the product in stock and would have provided it immediately had he been allowed to do so. I am left in the position of having to pay considerably more for a service that is significantly worse.

I have been told that Italy is a special case because collecting payments can be difficult. Although this is true for some institutions, it is not so for all, and problems of this sort could be provided for within the terms of a contract of sale. It also does not explain why the prices in

other European countries that do not have this problem remain considerably higher than those in the United Kingdom.

Would it not be to the benefit of both researcher and company if the supply of consumables and equipment was provided by single central European warehouses? This would guarantee a single uniform European price and a stock larger than that which could be carried by any individual national distributor. It is difficult to see why the efficient distribution service that exists in America could not be introduced into a Europe without frontiers. Failing this, the cosy local distributor arrangements should be terminated, allowing a scientist to shop around and purchase his reagents from the cheapest and best stocked European supplier even among the different distributors of a single producer.

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Palmer Station

SIR — On your recent article, “NSF hangs out wary welcome sign” (*Nature* 361, 106; 1993), I would like to correct Jeffrey Mervis’s statement that “visitors do not get a guided tour” of Palmer Station in Antarctica. Organized guided tours of Palmer Station have been provided by station personnel since 1988. The station tour is divided into two parts. The first is an outside tour that leads visitors, in groups of 25, though the centre of the station and ends at a display located just outside the Aquaria Laboratory. The second involves entering the primary building, BioLab, as it is called locally, and walking up to the kitchen and dining area. Refreshments are served and visitors have the opportunity to meet researchers, station support staff and the NSF-sponsored visiting artist. Each segment of the tour is 30 minutes long.

Visitors with special interests are generally accommodated if they request to see a part of the station that is not included in the regular tour. Ham radio operators are shown our communications centre and visiting physicians see the dispensary and X-ray room.

A four-hour visit requires 40 labour hours to support. Station personnel volunteer to serve as tour guides. The role of last year’s National Park Service representative was not to develop a new programme. The goal was to observe the existing tour programme and make recommendations for improvements.

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A strange discipline. . .

SIR — The review article "Strange kinetics" by Shlesinger *et al.*¹ explores the remarkable characteristics of complex, nonlinear processes evolving in space and time. The authors anticipate that a kinetic description of nonlinear dynamics will generate insights into a wide range of complex systems that evolve along interesting trajectories representing deterministic chaos. By coincidence it follows a thoughtful view² of the first 40 years of molecular biology. The spectacular advances along the trajectory of this field have been blemished in unexpected ways that escape an easy explanation. May not strange kinetics provide new insights into, or even remedies for, the shortcomings of molecular biology?

The shortcomings of molecular biology referred to may be side-effects of rapid progress: (1) Preoccupation with enumeration of molecular components without quantifying the processes involved. (2) A tendency by molecular biologists not to reflect on the significance of their data, but to focus on the next piece in one of the puzzles. (3) A reward system that encourages competition between colleagues with almost identical aims and skills.

How are nonlinear dynamical systems relevant? The evolution of a complex field such as molecular biology over a 40-year period would be expected to have a strange trajectory. Its behaviour and the new structures it generates would be at least as complex as those generated by the strange kinetics of simpler systems. Its trajectory would remain sensitive to the initial conditions under which it developed, themselves greatly influenced by theoretical considerations and by the technologies of the physical sciences. Only after a series of spectacular discoveries on the structure of DNA, the specificity of base pairing and the intricacies of gene expression did molecular biology mature around what might be called its own formalisms.

The observed specificity of molecular biology sets the field apart from the older disciplines of physiology and medicine, which often depended on a linear analysis of precisely measured flows in and out of a compartment defined by the investigator. In the kidney, for example, balance and clearance studies still serve to quantify its overall operations. Even at the epithelial and cellular levels, studies of transport have remained highly quantitative. When nonequilibrium thermodynamics was introduced to the study of irreversible processes, transport physiologists applied linear forms of analysis based on formalisms for near-equilibrium conditions^{3,4}.

These formalisms, however, were too restrictive for molecular biologists. The new formalisms of strange kinetics, on the

other hand, seem designed for nonlinear processes taking place far from equilibrium. They are rich enough to accommodate the degrees of freedom needed for biological systems. Their patterns, which antedate the genetic code, are in principle applicable to the molecular events and the time sequences of biology. Even if these formalisms are only occasionally of service, their development may contribute to the reflectiveness of the participants and, thereby, rescue molecular biology from its blemishes.

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1. Shlesinger, M. F., Zaslavsky, G. M. & Klafter, J. *Nature* **363**, 31–37 (1993).
2. Maddox, J. *Nature* **363**, 13 (1993).
3. Nicholas, G. & Prigogine, I. *Exploring Complexity* 54 (W. H. Freeman and Co, New York, 1989).
4. Andersen, O. S., Silveira, J. E. N. & Steinmetz, P. R. *J. gen. Physiol.* **86**, 215–234 (1985).

Molecular biology

SIR — I would like to comment on the article on the dark side of molecular biology¹, in which ideas already put forward² are developed further. It is true that molecular biology is a very powerful tool that gives rapid and simple answers — the gene is or is not present, is or is not altered — to complex questions and that many researchers are satisfied with these answers.

The problem arises because quantitative measurements, contrary to what is possible in the field of physical chemistry, are concerned with open dynamic systems in which responses to different stimuli are linked and nonlinear. The study of such systems entails the use of powerful computational methods and either the determination of wealth of physicochemical parameters or recourse to simplification and approximations. This is often considered too complex and useless by many, not to say most, molecular biologists, partly because the striking results obtained, for example, in the field of human health may lead people to think that the reductionist approach used is not only sufficient but also the only one necessary and valid in all cases. This situation also obscures the fact that the state of a nonlinear system is not only a function of the initial condition (among which are enzyme or receptor quantity and characteristics) but also of its history: a given system may end up at two different steady states, depending on the nature of the stimuli it received.

As you say, quantification is long and

difficult, too slow compared to the pace of the current descriptive race, and it also requires skills in the field of system dynamics. That is why the quantification effort comes mainly from chemical engineering laboratories, from people such as James Bailey³ and Gregory Stephanopoulos⁴, with the help of molecular biologists.

Let us hope that more people will be convinced of the importance of quantification, the only way to a global vision and a complete understanding of living systems.

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1. *Nature*, **363**, 13 (1993).
2. *Nature*, **355**, 201 (1992).
3. *Science*, **252**, 1668–1675 (1991).
4. *Science*, **252**, 1675–1681 (1991).

ESO's colony

SIR — After reading of the negotiations between the European Southern Observatory (ESO) and the Chilean government about the use of the observatories (*Nature* **363**, 384; 1993), as a Chilean scientist I could not avoid being struck by the similarities between the role of the ESO in Chile and the situation described by Joseph Conrad in *Nostramo*, his classic novel of colonialism in South America.

According to your report, the ESO in Chile appears to be the typical colonial enclave described by Conrad, acting independently of the local government, with policies that restrict the access of Chilean scientists to the laboratory and artificially lower the salaries of native workers and limit their professional advancement. This agency, moreover, expects the continuation of the advantageous concessions obtained from the Pinochet dictatorship. Nitrates, guano, silver and rubber may have been replaced by rights over land for laboratories and access to the sky, but the asymmetry of the relationship remains the same.

ESO's denial of guaranteed viewing time to Chilean astronomers, on the basis of their low numbers, is disingenuous and circular, as the only way to increase the number is to train more astronomers by, among other things, increasing their access to ESO. The fact that, after 30 years of ESO working in Chile, the Chilean astronomical community still remains small shows the unfairness of an arrangement that disregards the training of local scientists as a priority. It is difficult to imagine that any European country would tolerate the presence of a laboratory administered by Americans, to which the host country would be denied access.

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Religion and Christianity

SIR — It is rather unfair of Arno Arrak to criticize Brian Josephson's "concept of religion as an attempt to maximize human goodness" by pointing to the doctrinal extravagances engaged in, in the name of religion, by the Aztecs and Carthaginians (*Nature* 364, 276; 1993). It is not Quetzalcoatl, Moloch, or even the Buddha that is foremost in Josephson's thoughts. Josephson may use the word *religion* but what he means is *Christianity*, where human sacrifice is known to be frowned on. Arrak is on firmer ground when he points out that the "*sine qua non* of any religion is the existence of supernatural forces".

Josephson writes (*Nature* 362, 583; 1993) of the "function and fruitfulness" of religious (that is, Christian) belief. Functionally speaking then, what is the precise purpose (or Purpose) of the gods (in general or his own one in particular)? And whose purpose is served? Just what "fruitfulness" is in question, and whose fruitfulness is being addressed when scientists plump for superstition and irrationality and against the scientific approach to matters of reality, factuality and truth?

If we are frightened of the dark that awaits us then let us at least have the courage, and the honesty, to say so. If we must invent gods to grant us eternal life then let us have the moral integrity to say that this is what we are doing, and why.

Let us avoid the humbug, self-deception and philosophical cant with which we camouflage our terrors and hide our spiritual deceptions from ourselves and from others and call this *religion*.

Ralph Estling

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SIR — In science (and in other areas) it is bad practice to deduce average behaviour from extreme cases. A particularly colourful example is found in the correspondence from Arno Arrak, who picks the worst possible instance he can think of to justify his antagonism to religious belief.

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SIR — I am amused to read that the propensity to religious belief is all the fault of some bit of DNA (*Nature* 363, 389–390; 1993). Ernst Haeckel, Darwin's greatest evangelist, was of the same mind, boldly asserting that the propensity to mysticism is genetically derived from barbarians and savages. How, one wonders, can such ideas be regarded as evidence of rational thought?

I know — we all know — that the icon on my computer screen is not directly

related to the structure of a few semiconductors. It depends on me, my operations, on the electricity supply, on thousands of circuits and memory bits, on the properties of the screen, on magnetic fields and particle beams, on computer languages, and many other things. I also know that my computer is totally incapable of acquiring a propensity to any philosophy — it simply does not have the wherewithal to experience, to learn, to speculate, to make judgements. Maybe one day we will be able to simulate some of these higher faculties; but we can be sure that the processes involved will be of a different order of complexity from the structure of a protein, or a section of DNA.

The new genetic fundamentalism seems to me even more irrational than religious fundamentalism. The latter at least is a sincere attempt to find some meaning and purpose in life, even if, in the opinion of many, it is misguided. If everything we do, or believe, or aim for, is predetermined by bits of DNA, then what value is there in trying to analyse anything, and to make sensible judgements? What point can there be to the scientific quest?

Newton, Faraday and Maxwell, to name just the most obvious, provided us with vital insights that, however embarrassing to the reductionist, came out of their profoundly religious outlook. They were in fact obeying the specifically Christian injunction to "seek, and ye shall find; knock, and it shall be opened unto you". To assume that their science and their religion were distinct and unrelated is, in my opinion, not supported by any evidence or scholarship. Their remarkable work showed the intrinsic consistency in all physical processes — thus confirming the Christian injunction, and sustaining their belief in a meaningful universe.

Once again I would stress that this is an agnostic view. People of all faiths, and no faith, have contributed to the scientific culture. I write this letter to challenge the bias of your journal against religious and esoteric ideas, which is unfair to some of our greatest scientists, and unhelpful in the development of new ideas. In music, in art, in literature, and not least in science, we are all indebted to those of a mystical turn of mind.

John Evans

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Racial science

SIR — I recently visited the anthropology exhibits in the Museum of Natural History in Vienna. These constitute an understated but quite unequivocal endorsement of Nazi-like racial science. The first room

is arranged in continental displays. The main exhibits are a series of single skulls, each presented as representative of a racial type. For instance, in the African section were skulls labelled "Bushman", "Pygmy" and so on; in the European section skulls putatively representative of particular European types, including "Gypsy" but for some reason omitting Jews. In the next room, there is a case displaying two skeletons: one is labelled "Japanese", the other "Indonesian". The small evolutionary exhibit presents three skulls for comparison labelled australopithecine, chimpanzee and "Bushman": the evident message is that the Bushman skull represents a primitive human type.

My first impression was that this travesty had been set up in the 1930s or 1940s, for a considerable proportion of Austrian anthropologists were implicated in Nazi science, and some of the leading figures were enthusiastic Nazis. Perhaps, I thought, it had simply been neglected since, but I was assured that the exhibition had been set up comparatively recently. This is an outrageous display for a major European museum, and I hope that anthropologists throughout the world will join me in an urgent appeal to the museum to close the display, explain to the public why it is both scientifically indefensible and politically and morally offensive, and replace it with a modern exhibition which explains what is now known about human evolution.

Adam Kuper

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Genetic testing

SIR — I oppose Ron M. Kagan's letter headed "Justice is not egalitarian" (*Nature* 363, 578; 1993). Increasing use of genetic screening will demonstrate that everybody carries some genetic disability. If, according to Kagan's optimistic model, all these people would acquire "special skills in high demand" we would witness a cultural improvement of unprecedented scale.

Unfortunately, however, as our German poet Bert Brecht put it: "Yet, such conditions are not found out there" (*Doch die Verhältnisse, sie sind nicht so*). For this realistic reason, the thoughts of Benno Müller-Hill ought to be taken more seriously. As further reading, I recommend Paul R. Billings *et al.*: "Discrimination as a consequence of genetic testing" (*American Journal of Human Genetics* 50, 476–482; 1992).

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A home for the mighty mouse

With the aid of private health organizations and the Howard Hughes Medical Institute, the Jackson Laboratory will become the world's clearinghouse for transgenic and knockout mice — at a reasonable price.

At last, it is the decade of the mouse. Whereas once finding a mouse model for disease was a matter of serendipity, mice now can be created to a researcher's specifications. In the mid-1980s the "Harvard mouse", a transgenic creature that is susceptible to breast cancer, was developed under a research agreement with the DuPont Company that holds the patent.

Then there were 'knockout mice' — a variation on the theme of creating (rather than waiting for nature to reveal) research-friendly mice from the laboratory of Mario Capecchi at the Howard Hughes Medical Institute at the University of Utah, who discovered that mediating homologous recombination at the early S phase of the cell cycle could enable him to knock out or disable a gene at



will. One recent result is a mouse with developmental defects of the ear, cranial nerves and hindbrain, all because of a targeted disruption of the homeobox gene *Hox-1.6* (*Nature* **355**, 516–520; 1992).

Since then, there has been a veritable population explosion of transgenic and knockout mice. There is a mouse whose cystic fibrosis (CF) gene has been knocked out, making it the first animal model for that common genetic disease. An *Apoe* mouse that develops severe arterial plaque, even on a low-fat diet, is now available for use in research. And, perhaps most important, researchers, beginning with Allan Bradley's laboratory at Baylor College of Medicine in Houston, have introduced a null mutation for the *p53* gene in mice, showing that its absence predisposes mice to neoplastic disease by the age of six months (*Nature* **356**, 215–221; 1992).

As a result of these and other studies, says Kenneth Paigen, director of the Jackson Laboratory in Bar Harbor, Maine, "we are in the early stages of the most profound scientific revolution yet seen". Since 1929, the Jackson Laboratory has identified through careful screening and inbreeding some 1,700 genetically special strains of mice. But now, says Paigen, "with transgenic and knockout mice we suddenly have the ability to create tailor-made mammalian models of human

disease which offers the opportunity to study complex physiological phenomena, such as the nervous and immune systems, AIDS, and cancer, as never before. And we need to look at mice because they are a remarkable experimental surrogate for us, which is what medical research is really all about."

And so it seems that the scientific celebrity of the much studied *Escherichia coli*, of *Caenorhabditis elegans* (that intellectually beautiful worm), of the sturdy fruitfly and even the newly popular zebra fish is being eclipsed.

The decade of the mouse is here, but it did not take much prescience to see it coming. Ever since the recombinant DNA revolution made biological science a business by giving biologists products (such as drugs) and research tools (such as enzymes and mice) that they could sell, patent lawyers, stock traders and entrepreneurs have been avidly watching in the wings. In the mouse business, DuPont was first with the Harvard Myc mouse and a patent with a set of royalty payments and so-called 'reach-through' clauses affecting the use of progeny of breeding pairs that gave the traditional notion of the free exchange of scientific material among researchers a new meaning. Others followed suit, most notably GenPharm of Mountain View, California, which acquired from the Baylor laboratory the right to breed, sell (at around \$100 a mouse) and receive fees on the progeny of breeding pairs of Bradley's *p53* knockout.

"The GenPharm situation got everyone very heated", says Harold Varmus of the University of California at San Francisco (and director-designate of the US National Institutes of Health). At the Mouse Molecular Genetics meeting at Cold Spring Harbor in August 1992, he chaired what is politely referred to as a debate about what to do. "Ken Paigen gave an impassioned speech about the importance of free exchange", he recalls. Varmus then organized a more formal, less confrontational meeting on "Sharing laboratory resources" at the US National Academy of Sciences. Transgenic and knockout mice are expensive to create and costly to maintain (between \$50,000 and \$100,000 a year). They are therefore virtually out of reach of many researchers if they have to be purchased from commercial sources expecting (reasonably) to make a profit on their sales.

The NIH responded by putting out what it says is a once-only request for applications (RFA), offering to establish a national resource for transgenic [and knockout] ani-

mals. That grant should be awarded within a couple of months.

Meanwhile, Paigen started seeking funds from private health organizations for an 'induced mutant resource' at the laboratory in Bar Harbor. Remarkably, he struck a chord. The March of Dimes, which supports research on birth defects, made the first contribution, followed by the American Cancer Society, the American Heart Association, the Cystic Fibrosis Foundation and the Multiple Sclerosis Society. Now, in an act of unprecedented unity, these groups are urging larger charities, such as the Wellcome Foundation, to join them. Happily, the Howard Hughes Medical Institute made a commitment of \$1.2 million spread over three years and the resource is up and nearly running.

The Jackson Laboratory, with its proven capacity for breeding disease-free animals and for embryo cryopreservation, is distributing the CF mouse, and the Min mouse, which gets the equivalent of human colon cancer. And it is preparing to distribute a *p53* mutant from the laboratory of Tyler Jacks at the Massachusetts Institute of Technology (MIT). (To preserve a disease-free colony, breeding pairs of *p53*s from MIT will not be accepted. Only hysterectomy-derived pups will be admitted to the breeding colony.) Unlike the GenPharm *p53*, the Jax *p53* mouse will cost about \$60 a breeding pair, once there are enough of them. John Sharp, head of the induced mutant colony, says there is already a waiting list. The laboratory hopes to 'import' 50 different induced mutants in its first year of operation, eventually taking as many as 100 different breeding pairs. But even with solid funding and laboratory expansion, that may be the annual limit. A special admissions committee has therefore been established to evaluate candidate mice and select those that will be of greatest value to the scientific community. Getting one's mouse into the Jax colony may one day be as competitive as getting a child into an exclusive nursery school.

With subsidies from private foundations and health charities, bolstered by underwriting from the NIH (if the laboratory wins the grant), the not-for-profit Jackson Laboratory can afford to make these invaluable mouse resources affordable to others. In the process, it will not only advance the cause of science but also bring back an earlier day when science and profit did not necessarily go hand in hand.

Barbara J. Culliton

Two ears and two eyes

John D. Pettigrew

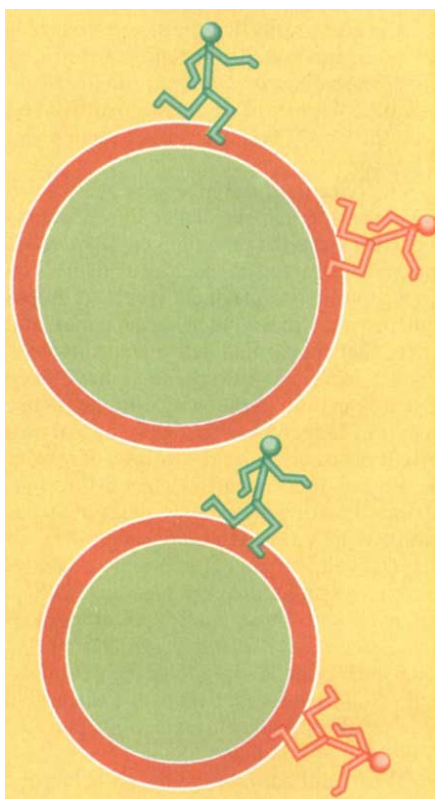
NEITHER the distance of a visual target nor the location of a sound source can be determined directly from the respective sensory epithelium, so predators make the higher-order computation by comparing inputs from the two eyes or the two ears. Owls use both binocular and binaural computations to guide prey capture and have added much to our understanding of the neural mechanisms involved (see for example refs 1, 2). New work by Wagner and Frost, reported on page 796 of this issue³, shows an interesting commonality in the underlying neural strategies used by the binocular depth system and the binaural sound localization system.

The fundamental problem for both the binocular and binaural systems is the correspondence, or matching, problem: namely to identify the pair of image segments, one on each sensory epithelium, that belongs to the same visual target or sound source. Once this problem is solved, then small differences between the paired stimuli can be measured to estimate the higher-order dimensions of visual depth or sound location. There is a fundamental incompatibility between these two operations, in that matches cannot avoid some measure of similarity that might lose information about the differences, and vice versa.

Perhaps for this reason, all systems so far studied, whether in animals or machines, make very early comparisons between raw descriptions of the neural image from both eyes or both ears, before proceeding with more complex analysis that might result in the loss of information useful for the match^{4,5}. It was this extreme earliness in the appearance of binocular specificity in the visual pathway that took investigators by surprise and created controversy in the 1960s⁶. Although humans have extraordinary flexibility in their choice of 'primitives' as a basis for matching the neural images from each side⁴, it is generally agreed, on the basis of neurophysiological recording, that edge orientation is one of the basic features used by many species for the matching operation in binocular visual systems, while sound frequency is the basic feature matched in binaural auditory systems³.

A visual stimulus can have a frequency too, as in a grating with a spatial frequency that is analogous to the sound frequency. Repeated stimuli of this kind, visual or auditory, suffer a similar ambiguity in phase when images from both sides are compared. Resolving ambiguities of interaural and interocular phase is an important problem for the nervous system, and the new work on the owl helps us to understand how it is achieved.

The phase ambiguity problem is depicted in the figure, inspired by Mark Konishi, which shows two runners engaged in a race of many laps around a circular track. A snapshot of the race would be ambiguous, because an apparent leader might be about to be lapped by the real winner. This is analogous to the owl's problem of trying to decide whether a regularly repeated stimulus on one side is



The phase ambiguity problem, exemplified by a snapshot of two runners on a circular track. Who is leading? Is it red, or is green about to lap red? This ambiguity can be resolved if another snapshot is taken of the same race, but on a smaller racetrack. Because red has an increased lead on the smaller (higher frequency) track compared with the larger track, we can infer that red must be leading. In a similar way, the brain can resolve ambiguities in the phase differences between images from both eyes or both ears by using a variety of different frequencies.

ahead or behind its partner on the other side. The owl's nervous system solves this problem by arranging for the race to be run simultaneously on a number of different sized race tracks (that is, at a number of different frequencies). By pooling the information from all races (frequencies) it is possible to remove the ambiguity and to obtain the exact margin by which the image on one side leads the other⁷⁻¹⁰.

The binocular neurons in the owl's visual cortex studied by Wagner and Frost use the same kind of strategy for resolving the phase ambiguities in the visual system. These 'disparity tuned' binocular neurons also pool information from different spatial frequencies, choosing only those inputs with phase relations that are consistent with the same depth plane. In keeping with the use of the term 'characteristic delay' for the time shared in common by the phases of all the sound frequencies coming from a particular location, they use 'characteristic disparity' to describe the angular difference between the two eyes that would produce the phase relations observed between all the different spatial frequencies. In this way all the appropriate combinations of spatial frequency and interocular phase that correspond to one depth plane are 'glued' together.

As with space-specific neurons in the auditory system, it is not clear how constraints are brought to bear on the system to find the right set of phase relations to wire up the depth-specific neuron. There are reports of spatial frequency-selective neurons that appear to recognize harmonic relations in the cat's visual cortex¹¹, so the visual system might also use the harmonic constraint (that harmonically related spatial frequencies should show harmonically related phase differences if they are coming from the same depth plane). Alternatively, or in addition, the well-known developmental plasticity of these binocular visual connections^{12,13} might permit the use-dependent assembly of the appropriate set of phase relations, as well as their subsequent modification as the head and eyes grow.

This model of disparity processing by the owl is a little different from recent models of the cat's visual cortex, where anti-phase relationships have been proposed between the two eyes¹⁴. This could be written off as a species difference. But the striking similarities observed between

1. Konishi, M. *Sci. Am.* 66–73 (April, 1993).
2. Pettigrew, J. D. *Proc. R. Soc. B* **204**, 435–454 (1979).
3. Wagner, H. & Frost, B. *Nature* **364**, 796–798 (1993).
4. Julesz, B. *The Foundations of Cyclopean Perception* (Univ. Chicago Press, 1971).
5. Pettigrew, J. D. in *Visual Neuroscience* (eds Pettigrew, J. D., Sanderson, K. J. & Levick, W. R.) 777–787 (Cambridge Univ. Press, 1986).
6. Bishop, P. O. & Pettigrew, J. D. *Vision Res.* **26**, 1587–1600 (1986).
7. Takahashi, T. & Konishi, M. *J. Neurosci.* **6**, 3413 (1986).
8. Wagner, H., Takahashi, T. & Konishi, M. *J. Neurosci.* **7**, 3105–3116 (1987).
9. Schreiner, C. E. & Langner, G. *J. Neurophysiol.* **60**, 1823–1840 (1988).
10. Knudsen, E. I. & Brainard, M. S. *Science* **253**, 85 (1991).
11. Pollen, D. A., Andrews, B. A. & Feldon, S. E. *Vision Res.* **18**, 665–682 (1978).
12. Hubel, D. H., Wiesel, T. N. & LeVay, S. *Phil. Trans. R. Soc. B* **278**, 377–409 (1977).
13. Pettigrew, J. D. & Konishi, M. *Nature* **264**, 753–754 (1976).
14. DeAngelis, G. C., Ohzawa, I. & Freeman, R. D. *Nature* **352**, 156–159 (1991).
15. Mayhew, J. E. W. & Longuet-Higgins, H. C. *Nature* **297**, 376–378 (1982).
16. Maske, R., Yamane, S. & Bishop, P. O. *Proc. R. Soc. B* **229**, 257–276 (1986).

the fundamental features (as opposed to 'neural embroidery') shared by the mammalian and avian neural apparatus for prey acquisition suggest that class chauvinism is not the answer here. The Wagner and Frost model is more appealing because it can be reconciled with the needs of the matching operation (anti-phase operations in each eye are the very antithesis of matching). Perhaps a role for the anti-phase operators can be found in other binocular phenomena, such as rivalry, lustre or the sharpening of disparity-tuning curves.

The appropriateness of Wagner and Frost's model is confirmed by a consideration of binocular, end-stopped cells, a class of cells that plays a special role in binocular depth judgements about horizontal contours. Horizontal contours have always posed a potential problem in binocular vision, because, unless terminated, they do not provide information about the horizontal disparities that are

conventionally thought to be of major importance for depth judgements. Recent work, reaffirming old studies showing that vertical disparities can be used to provide depth information, tends to reduce this problem¹⁵. More importantly, horizontally oriented end-stopped cells are beautifully tuned for horizontal disparities¹⁶. These cells present a limiting case in the present discussion about the use of phase information, in that the termination that they signal is a square wave containing all spatial frequencies at the same phase. The sharp disparity tuning of end-stopped cells, combined with the new results in the owl, reinforce the view that characteristic delay and characteristic disparity are powerful concepts that reflect a basic strategy to 'disambiguate' phase information when neural images meet. □

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GALAXY EVOLUTION

Once upon a time in the Galaxy

Leo Blitz

THE history of the Milky Way is written in the distribution of its stars, and its formation is traced by its oldest star clusters, the globular clusters. Attempts to decipher these distributions have led to a proliferation of proposed histories of the Galaxy. Now Sidney van den Bergh argues that a *mélange* of these proposed histories is required to explain the distribution of globular clusters (*Astrophys. J.* **411**, 178; 1993). One group of clusters seems to be the result of the cannibalization of a small companion galaxy in which the clusters had already formed; another appears to be the result of the rapid collapse of the protogalactic halo.

Globular clusters (see photograph) are among the oldest objects in the Milky Way, and because the clusters that we observe have not been greatly disturbed since their formation, they provide a measure of the conditions at the time of their formation roughly 10–15 billion years ago, during the early history of the Universe. The clusters themselves have lost virtually all traces of their own formation histories, but have nonetheless left footprints that enable astrophysicists to learn about the development of the Milky Way system.

For example, the abundance of elements heavier than helium varies greatly among the globular clusters. The envelopes of the clusters associated with the galactic halo have long been known to be deficient in these elements, which are thought to be produced only deep inside stars. This observation spawned the idea

that the globular clusters in the halo formed early, and that subsequent generations of stars coalesced from clouds of gas enriched with the ejecta from the supernova explosions of stars formed at the earliest epoch.

Van den Bergh identifies three distinct populations of clusters using a new classification scheme he devised. He separates clusters according to the abundance of

iron in the stellar atmospheres, and a parameter based on the numbers of evolved blue, red and variable stars in a cluster. Because the relative numbers of these stars is determined by the evolutionary state of the cluster as a whole, it is possible to use the parameter to identify clusters that formed at the same epoch, and others that formed over a more extended period of time.

The clusters with high iron abundancies are grouped together and are found to be strongly concentrated towards the bulge and disk of the Galaxy, as has been known for some time. The iron-poor clusters are divided into two subgroups: coeval clusters formed within about a billion years of one another, and temporally extended clusters formed over a period of several billion years. The clusters formed coevally are the oldest of the three populations, and are found closer to the centre of the Galaxy, on average, than the more temporally extended clusters. Van den Bergh argues that the distribution of clusters thus suggests that the inner part of the protogalaxy collapsed rapidly, and was the first distinct part of the Milky Way to form. The outer clusters formed later and in some other way.

A clue to how the outer clusters formed comes from their inferred motions about the Galactic Centre. In certain fortuitous cases, the measured position and radial velocity of a cluster make it possible to determine whether it is orbiting in the same sense or the opposite sense to the rotation of the galactic disk. It is also possible in some cases to determine whether the orbits of a group of clusters are primarily circular or radial. Presumably, if all the clusters formed from a



Optical photograph of M22, a globular cluster in the constellation Sagittarius.

Science Photo Library

single protogalaxy, conservation of angular momentum requires that on average the clusters have prograde orbits, orbits in the same sense as that of the disk. But van den Bergh finds that for the iron-poor clusters, prograde orbits predominate only for the clusters formed coevally; retrograde orbits predominate for the clusters formed over a more extended period. Where the shape of the orbit could be determined, he finds that those further than the Sun from the Galactic Centre (mainly those formed over an extended epoch) have radial orbits. Furthermore, the clusters with circular orbits tend to have larger diameters than those with radial orbits, presumably because the radial orbits bring them closer to the Galactic Centre and tidally strip off more of the outlying stars.

Putting these deductions together, van den Bergh concludes that the more outlying clusters, which are more enriched in iron than the oldest group, formed in a separate dwarf galaxy, perhaps similar to the Large Magellanic Cloud. This ancestral galaxy had a retrograde orbit with respect to the rotation of the Milky Way, and was devoured by it as it fell into the Galaxy. Evidence for similar mergers in other galaxies has become common in the past five years, and such an occurrence in the Milky Way would not therefore have been a particularly unusual event. The radial, retrograde orbits of the clusters would then be the fossils of the motion of the ancestral galaxy, and would occur naturally if it had an orbit slightly counter to that of the Milky Way.

The formation of the globular cluster system, presumably mirroring that of the Milky Way as a whole, has therefore had at least three distinct epochs. The earliest is the rapid collapse of the inner halo signified by the coeval globulars. The capture and dissolution of the ancestral dwarf galaxy was next and most probably preceded the formation of the disk, the bulge and the iron-rich globular clusters. Independent evidence for the ordering of the latter two groups comes from the quiescence of the galactic disk which requires that no galaxy as massive as the Large Magellanic Cloud has collided with the disk since its formation (G. Toth and J. P. Ostriker, *Astrophys. J.* **389**, 5–26; 1992).

For this formation history to be truly compelling, one would like to see corroborating evidence from the stars in the galactic halo that are not in clusters. Nevertheless, it is surprising that one can still learn fundamentally new things about the history of the Galaxy from classical observations of so well studied a set of objects as the globular clusters. □

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The response to *vagusstoff*

Henry A. Lester and Nathan Dascal

THE molecular cloning of all the major components in the transduction pathway described in Otto Loewi's famous experiments on isolated frog hearts¹ is now complete with the publication of a letter by Kubo *et al.*² on page 802 of this issue. Loewi cannulated two hearts and connected the effluent from heart A to the input of heart B. He then stimulated the vagus nerve to heart A; in agreement with previous experiments, heart A ceased beating for several minutes. But when the

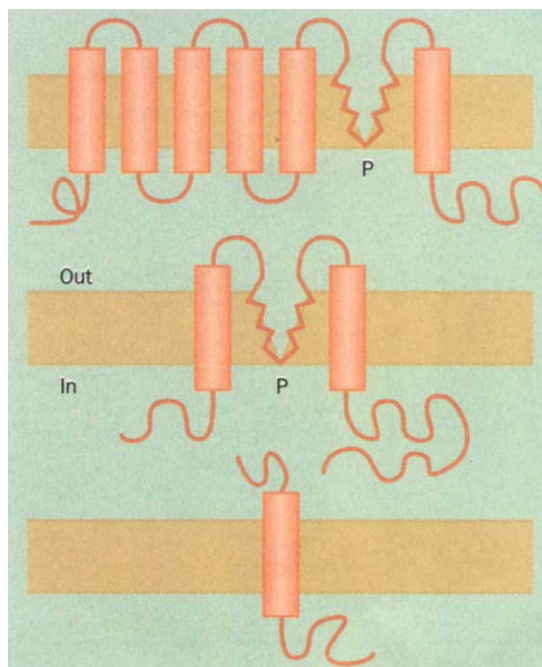
protein effectors such as adenylyl cyclase, phospholipase C, or retinal rod phosphodiesterase. This hypothesis is strengthened by the observation that excised inside-out patches from oocytes expressing GIRK1/KGA show single inwardly rectifying channels identical to those of frog heart when the G proteins are appropriately activated by 7-helix receptors and/or nonhydrolysable GTP analogues^{1,3}. Additional information may come from biochemical measurements on

direct interactions between G proteins and GIRK1/KGA. GIRK1/KGA resembles two other recently cloned inwardly rectifying channels in that it has two transmembrane helices and a P region (see figure); however, the newly sequenced cDNA shares no detailed homology with the other known G-protein effectors.

If the GIRK1/KGA channel is indeed a direct G-protein effector, then the single-molecule resolution of electrophysiology may enable sensitive new assays for the G protein-effector interaction to be devised. For instance, an important scheme for K⁺ channel inactivation, the ball-and-chain model⁵, bears at least a mechanical resemblance to the postulated mechanisms in which G proteins remove inhibitory domains or subunits that are part of the effector protein, but which hinder access to the catalytic site⁶. Candidates for such gating domains on GIRK1/KGA

include the putative N- and C-terminal cytoplasmic domains that are not homologous to other inwardly rectifying K channels. The N-terminal region includes a lysine/arginine-rich stretch like that on the 'Shaker ball' and there is an intriguing nearby sequence with several prolines. We don't know whether GIRK1/KGA is a tetramer, like the Shaker family of voltage-dependent K⁺ channels, but some tantalizing results suggest that activation requires three or four G-protein α -subunits acting cooperatively⁷.

Which G protein activates GIRK1/KGA, and does activation proceed through the α -subunits, $\beta\gamma$ dimers and/or eicosanoids? It will be a while before this is sorted out. G_i/G_o α -subunits are likely to play a major part in the pathway⁸, but under some circumstances pertussis-toxin-insensitive G proteins can substitute⁹, and purified $\beta\gamma$ subunits¹⁰ also



Presumed topology for individual subunits of the known plasma membrane K⁺ channels. Top to bottom: Shaker class voltage-gated channels, inward rectifiers and minK¹³. The P region is thought to line the conducting pore.

perfusate reached heart B, it too stopped beating. Evidently a substance (*stoff*) released from the vagus nerve was capable of stopping the heart. The *vagusstoff* was acetylcholine, acting on muscarinic m2 receptors in the atrium to activate potassium channels: Loewi's report¹ proved the existence of chemical synaptic transmission.

The newly cloned potassium channel complementary DNA, termed GIRK1 (ref. 1) or KGA (ref. 3), has been eagerly awaited for several reasons. First, no soluble intermediate is required in the pathway from 7-helix receptors to heterotrimeric G proteins to the channel⁴. In a conservative interpretation of this observation this pathway is 'membrane-delimited', but a more daring interpretation has GIRK1/KGA, in addition to comprising a channel, as a G-protein effector, fully analogous to other G-

activate at least as well as $G\alpha_i$. One fascinating aspect of this pathway is its speed¹¹: activation proceeds with a time constant of about 300 ms and deactivation with a time constant of around 1 s, yet isolated G_i and G_o subunits have a much lower rate of GTPase turnover. Perhaps the channel itself is a GTPase-activating protein; this can now be tested.

Neuroscientists will be addressing the possibility that GIRK1/KGA, which is expressed in the brain³, or homologous channels comprise the inwardly rectifying, inhibitory K^+ conductance evoked in neurons or neuroendocrine cells by many 7-helix receptors that couple to G_i or to G_o . These receptors include muscarinic m2 and m4, various serotonin 5HT-1 receptors, μ or δ opioid, α_2 adrenergic, somatostatin, $GABA_B$, and D2 and other dopamine receptors¹². Although G_i was first named for its ability to inhibit adenylyl cyclase, this action may be less important than its ability to activate K^+ channels and inhibit Ca^{2+} channels. Both actions would inhibit transmitter release from presynaptic terminals.

Will GIRK1/KGA or another inward K^+ rectifier channel prove a useful target for therapeutic drugs? Cardiovascular pharmacology is a rather mature field, and it is unlikely that new drugs could provide better control of atrial fibrillation. As for neuroscience-based therapies, we need to know more about the diversity of this family. If specific members are found, for instance, that participate in the inhibition of neurons by the opioid or α_2 receptors, then a specific and direct activator of such a channel would by-pass the pathways that lead to downregulation and phosphorylation of the 7-helix receptors involved. Such a drug might have unique properties for neuropsychiatric or analgesic application. K^+ channels have entered a fascinating new phase of research. □

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New spin on ancient climate

James F. Kasting

WHY didn't the Earth freeze over in its infancy, when the Sun was fainter than it is today? This 'faint young Sun' problem has a number of proposed remedies¹⁻³, high atmospheric concentrations of carbon dioxide being perhaps the most attractive^{2,4}. Now Jenkins *et al.*⁵ have added another: reduced solar luminosity in the past could have been at least partially offset by a lower surface albedo caused by decreased land area and cloud cover on a rapidly spinning young Earth.

The new study is one of only a handful of calculations relating to Precambrian climate that have been performed using atmospheric general circulation models. The model used in this case was a variant (CCM0A) of the community climate model developed at the National Center for Atmospheric Research in Boulder, Colorado. A list of things that the model did not include shows that our ability to simulate palaeoclimate is still rather limited. The model has no ocean heat capacity or heat transport, no seasonal cycle, and — although it was being used to study the Precambrian era — assumes no change in solar luminosity. (The Precambrian covers the period from 4.5 to 0.6 billion years ago, during which solar luminosity increased from about 70 to 95 per cent of its present value, according to standard solar evolution models⁶.) By restricting the scope of the present study, however, the authors were able to isolate the effects of two factors, land area and rotation rate, that are thought to have been different on the Precambrian Earth.

A variety of sedimentological and geochemical arguments, summarized in ref. 7, suggest that the continents were originally much smaller than they are today. Jenkins and colleagues extended this argument to its logical extreme by assuming a completely ocean-covered planet. The early Earth is also thought to have been rotating faster than today. (A decrease in rotation rate over time is an inevitable consequence of the tidal evolution of the Earth–Moon system.) Following Walker and Zahnle⁸, Jenkins *et al.* assumed that 4 billion years ago, the days were only 14 hours long.

Making these two changes in the input parameters to the community climate model resulted in a noticeable increase in the predicted global average surface temperature, T_s . Replacing land with water had the largest effect: T_s increased by 4 °C, mostly as a consequence of the lower albedo of water. Decreasing the Earth's rotation period to 14 hours resulted in an additional 1.5 °C of warming. The direct cause of this warming was a 20 per cent decrease in cloud cover associ-

ated with lower relative humidity and weaker vertical motions at middle to high latitudes. The faster rotation rate also resulted in other dynamical changes in the atmosphere, including a weakening of the tropical Hadley circulation (the large, convective cells that carry heat poleward from the Equator to about 25° latitude), an increase in the Equator-to-pole temperature gradient, and a tendency towards doubling of the subtropical jet stream.

How significant are these changes for the 'faint young Sun' problem? Calculations with a one-dimensional, radiative-convective, climate model⁹ predict that a 30 per cent decrease in solar luminosity would result in about a 30-degree drop in T_s from the present value of 15 °C. The greenhouse effect of CO_2 increases T_s by about 10 degrees for each factor of 10 increase in CO_2 , so a 30 per cent decrease in solar luminosity could have been offset by roughly a thousand-fold increase in atmospheric CO_2 concentrations. Smaller increases in atmospheric CO_2 might be allowed if early Precambrian surface temperatures were lower than today (unlikely, given the lack of evidence for glaciation), or if other infrared-active gases, methane for example, contributed to the greenhouse effect.

The changes in land area and rotation rate considered by Jenkins *et al.* combine to produce a surface warming of about 5 degrees, which corresponds to a threefold increase in atmospheric CO_2 according to ref. 9. Thus, including these factors in one's analysis should diminish the required CO_2 enhancement at 4.5 billion years ago to a factor of around 300 above the present level, that is, to a CO_2 partial pressure of roughly 0.1 bar. This result is not particularly significant by itself, as geochemical models predict that atmospheric CO_2 should have risen to whatever level was necessary to maintain liquid water and allow silicate weathering to proceed⁴. Lower CO_2 concentrations might, however, be easier to reconcile

- Loewi, O. *Pflügers Arch. Ges. Physiol.* **189**, 239–242 (1921).
- Kubo, Y., Reuveny, E., Slesinger, P. A., Jan, Y. N. & Jan, L. Y. *Nature* **364**, 802–806 (1993).
- Dascal, N. *et al. Proc. natn. Acad. Sci. U.S.A.* (in the press).
- Pfaffinger, P. J., Martin, J. M., Hunter, D. D., Nathanson, N. & Hille, B. *Nature* **217**, 536–538 (1985).
- Hoshi, T., Zagotta, W. N. & Aldrich, R. W. *Science* **250**, 533–538 (1990).
- Artemyev, N. O., Rarick, H. M., Mills, J. S., Skiba, N. P. & Hamm, H. E. *J. biol. Chem.* **267**, 25067–25072 (1992).
- Ito, H. *et al. J. gen. Physiol.* **98**, 517–533 (1991).
- Brown, A. M. & Birnbaumer, L. A. *Rev. Physiol.* **52**, 197–213 (1990).
- Dascal, N. *et al. Proc. natn. Acad. Sci. U.S.A.* **90**, 6596–6600 (1993).
- Ito, H. *et al. J. gen. Physiol.* **99**, 961–983 (1992).
- Karschin, A. *et al. Proc. natn. Acad. Sci. U.S.A.* **88**, 5694–5698 (1991).
- North, R. A. *Brit. J. Pharmac.* **98**, 13–28 (1989).
- Takumi, T., Ohkubo, H. & Nakanishi, S. *Science* **242**, 1042–1045 (1988).

- Sagan, C. & Mullen, G. *Science* **177**, 52–56 (1972).
- Owen, T., Cess, R. D. & Ramanathan, V. *Nature* **277**, 640–642 (1979).
- Rossow, W. B. *et al. Science* **217**, 1245–1247 (1982).
- Walker, J. C. G., Hays, P. B. & Kasting, J. F. *J. geophys. Res.* **86**, 9776–9782 (1981).
- Jenkins, G. S., Marshall, H. G. & Kuhn, W. R. *J. geophys. Res.* **98**, 8785–8791 (1993).
- Gough, D. O. *Solar Phys.* **74**, 21–34 (1981).
- Walker, J. C. G. *Origins Life* **16**, 117–127 (1985).
- Walker, J. C. G. & Zahnle, K. J. *Nature* **320**, 600–602 (1986).
- Kasting, J. F. *et al. J. atmos. Chem.* **1**, 403–428 (1984).
- Holland, H. D. *The Chemical Evolution of the Atmosphere and Oceans*, 179–186 (Princeton Univ. Press, 1984).
- Ahrens, T. J. *et al. in Origin and Evolution of Planetary and Satellite Atmospheres* (eds Atreya, S. K., Pollack, J. B. & Matthews, M. S.) 328–385 (Univ. Arizona Press, 1989).
- Kasting, J. F. & Ackerman, T. P. *Science* **234**, 1383–1385 (1986).

with evidence for a relatively uniform degree of chemical weathering of surface rocks over geological time¹⁰.

Paradoxically, the changes considered by Jenkins *et al.* could actually have had the opposite effect. Walker⁷ showed that decreased land area on the early Earth should have had a profound effect on the global carbon cycle. Most of the carbon now present at the Earth's surface is stored in carbonate rocks (limestone and dolomite) on the continents. If the continents were initially absent, and if much of the Earth's carbon were delivered to the surface early by impact degassing¹¹, this carbon would have to have been partitioned between the atmosphere, ocean and sea-floor sediments. The sea floor is continually being created and destroyed as part of the plate-tectonic cycle and so is not a stable, long-term reservoir for carbonates. Furthermore, the silicate weathering process that removes CO₂ from the current atmosphere would have been very inefficient on an ocean-covered early

Earth. From these considerations, Walker concluded that the atmosphere might have contained as much as 10 bars of CO₂ until the continents began to emerge. Such a dense-CO₂ atmosphere could have produced surface temperatures of around 85 °C, despite the faintness of the young Sun¹².

All of this goes to show that predicting climate and atmospheric composition on the early Earth is a complicated problem. Calculations such as those of Jenkins *et al.* are interesting, but need to be considered in the context of an interlinked atmospheric-hydrological-geochemical system. Although the new calculations permit lower amounts of atmospheric CO₂ in the distant past, the more likely consequence of the postulated changes is a warm, dense palaeoatmosphere. □

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PLATE TECTONICS

Orbital dates and steady rates

Richard G. Gordon

ARE the Earth's plate motions steady, or do they change over millions of years, hundreds of thousands of years, or shorter intervals? The motion of some plates has been nearly constant over the past 5 million years, according to Douglas Wilson, whose results are reported on page 788 of this issue¹. But earlier investigation of the same question by Vogt² indicated that the rates of sea-floor spreading have been 22 per cent faster over the past million years than in the preceding million-year interval. Which answer is likely to be correct?

Both Vogt's and Wilson's histories are drawn from sea-floor spreading, where two lithospheric plates pull apart and the gap is constantly refilled by fresh material upwelling from the mantle. Mid-ocean ridges, the topographic sign of such spreading centres, are thus flanked by lithosphere whose age increases with distance from the ridge. Reversals of the Earth's magnetic field leave adjacent stripes of sea floor magnetized in opposing directions or polarities. These contrasts in sea-floor magnetization cause subtle variations or 'anomalies' in the intensity of the magnetic field measured near the sea surface. The anomalies are identifiable and correlatable across mid-ocean ridges worldwide. Their spacing across a mid-ocean ridge, along with their age, permits rates of sea-floor spreading to be determined.

The accurate determination of any geological rate requires accurate dating.

Vogt's results were determined using timescales developed from integrated studies of the magnetic polarity of igneous rocks on land and of the potassium/argon ratio, which can be used to estimate ages of geomagnetic reversals as old as 4 million years. Such timescales were fundamental 25–30 years ago in establishing the reality of sea-floor spreading and have long been the accepted standard, but recently have been suggested to need unexpectedly large revision.

Palaeoclimatologists can take the credit for this. Their idea was to calibrate climate indicators in the sea-bed sediments against changes in the Earth's orbital parameters that might have affected the amount of sunlight reaching the surface. The parameters — specifically the tilt or obliquity, the precession of the equinox, and the eccentricity — are calculated from numerical solutions of the motion of the Earth and other planets about the Sun in the past 5 million years. The tilt (the angle that the equatorial plane makes with the ecliptic) has a quasi-period near 41 kyr; the precession of the equinox as modulated by the eccentricity of the Earth's orbit has quasi-periods near 19 and 23 kyr (and between).

A good recorder of past climate conditions, reflecting variations in global ice volume, is the ratio of stable isotopes of oxygen, ¹⁸O and ¹⁶O, in the remains of fossil micro-organisms. From the high-resolution isotope record of Ocean Drilling Project site 677, Shackleton

*et al.*³ estimated ages consistently 5–7 per cent older than previous estimates for geomagnetic reversals since the Matuyama-Gauss reversal, which they date at 2.60 Myr ago. Independently, Hilgen^{4,5} correlated the calculated orbital record with the cyclic pattern of sapropels, which are brownish, often well-laminated, discrete layers enriched in organic carbon and are found in sequences of marine sediments. He derived a timescale for the past 5.23 Myr similar to that of Shackleton *et al.* over the past 2.60 Myr. Again his age estimates for geomagnetic reversals are 4–10 per cent greater than previously accepted. These revisions to the timescale have raised some eyebrows — the K-Ar dates had been thought to be far more accurate than this.

Baksi *et al.*⁶ critically tested the age of the most recent reversal (the Brunhes-Matuyama) by dating samples from a sequence of lavas known to record the transitional directions of the palaeomagnetic field during the reversal itself. K-Ar dating of samples stratigraphically near this reversal in other field areas had indicated an age of 730 kyr, whereas the age indicated from orbital-climate calibration is 780 kyr. Their results, obtained from ⁴⁰Ar/³⁹Ar incremental heating studies, gave a date of 783 ± 22 kyr (95 per cent confidence limits), decisively excluding the earlier age estimate and consistent with that indicated by the orbital-climate calibration.

Wilson uses the orbital-climate calibrated timescale together with high-precision plate-rotation solutions for the plate pairs across five mid-ocean ridges. If the recent revisions to the timescale were simply a fixed fractional increase in age, then Wilson would have found a pattern of significantly changing rates of sea-floor spreading identical to that found by Vogt². Instead, any consistent change is small, at least for the 8 to 11 tie points defined by the reversal record of sea-floor magnetic anomalies across each of the five mid-ocean ridges analysed. Wilson's best data at the fastest mid-ocean ridge permit the amount of sea-floor produced in a 1-Myr interval to be measured with an accuracy of about 1 per cent (to ± 1.5 km within 150 km), which provides a strong test of the consistency of a constant rate of sea-floor spreading with the new timescale.

There are, of course, two assumptions under trial here — first, that the orbital-climate calibrated timescale is correct, and second, that the sea-floor spreading rates are constant. In so far as the orbital-climate calibrated timescale is correct, Wilson's results show that any globally synchronous acceleration is no more than 3 per cent, much smaller than the 22 per cent estimated by Vogt. The main exception seems to be the spreading rates inferred before and after the Matuyama-

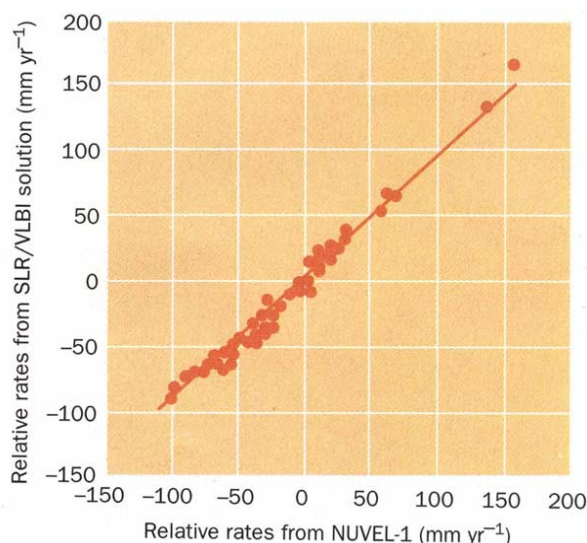
Gauss reversal. Either three plate pairs separated faster before and slower after this reversal or, as Wilson asserts, the age of the reversal is 10 to 20 kyr younger than indicated in the new timescales^{3,4}. It is unclear how these small differences can be independently resolved with available techniques.

If one accepts the implication that plate motions are very steady over the past several million years, it is natural to ask how true this is over briefer timescales. The expanding data from space geodesy, mainly from very-long-baseline interferometry (VLBI) and satellite laser ranging (SLR), place strong constraints on plate velocities averaged over the past several to dozen years⁷⁻⁹ and are similar to velocities calculated from the NUVEL-1 global model of plate velocities averaged over the past approximately 3 Myr (ref. 10). The correlation coefficient between space geodetic and NUVEL-1 rates is 0.994, but the space geodetic rates are slower by 6 ± 1 per cent (see figure¹¹). The new timescale³ indicates that the ages assumed in calibrating NUVEL-1 were about 4.5 per cent too young, so it gives velocities 4.5 per cent too fast, which leaves a marginally significant difference of about 1.5 per cent between plate-tectonic rates averaged over years and those averaged over millions of years. There are many possible explanations for this small remaining difference.

The implications of the combined results from precise estimates of sea-floor spreading, the revised geomagnetic reversal timescale and space geodesy indicate that plate motions are very steady, to within a few per cent, over a timescale that ranges from several years to several millions of years. Steady motion of plate interiors may seem surprising in light of the episodic motion associated with earthquakes at plate boundaries. However, outside the zone of elastic strain accumulation surrounding locked faults between earthquakes, the steadiness is an expected consequence of the dynamic equilibrium of driving forces (buoyancy forces and pressure gradients) with viscous resistive forces in the highly damped convective system of the solid Earth. The driving forces of a tectonic plate, and hence its velocity, are expected to be constant in so far as the geometry and thermal structure of a plate, its boundary, and any attached subducted slab are unchanged.

Wilson's work places tentative limits on

how long steady motion persists, as several plate pairs have been steady within 3 per cent or less for intervals of 2 to 4 Myr (possibly longer in one case). Similar analyses of other plate pairs could take this further, and might place limits on how quickly plate velocities readjust during their apparently infrequent accelerations. Studies of marine magnetic anomalies cannot, however, tell us the shortest interval over which plate motions are steady. Perhaps continuous monitoring of plate



Correlation plot showing how closely the plate motion rates derived from space geodesy, averaged over about 4 to 12 years, compare with rates averaged over about 3 Myr. Space geodetic results are based on 149 lines between 20 sites far from plate boundaries. After ref. 11.

motions using Global Positioning System geodesy will eventually determine whether plates move steadily at timescales even shorter than several years, as would be expected from our understanding of the physics of plate motion. □

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1. Wilson, D. S. *Nature* **364**, 788–790 (1993).
2. Vogt, P. R. in *The Geology of North America*, Vol. M, *The Western North Atlantic Region* (eds Vogt, P. R. & Tucholke, B. E.) 405–425 (The Geological Society of America, Boulder, 1986).
3. Shackleton, N. J., Berger, A. & Peltier, W. R. *Trans. R. Soc. Edin.: Earth Sci.* **81**, 251–261 (1990).
4. Hilgen, F. J. *Earth planet. Sci. Lett.* **104**, 226–244 (1991).
5. Hilgen, F. J. *Earth planet. Sci. Lett.* **107**, 349 (1991).
6. Baksi, A. K., Hsu, V., McWilliams, M. O. & Farrar, E. *Science* **256**, 356–357 (1992).
7. Clark, T. A. et al. *J. geophys. Res.* **92**, 12741–12750 (1987).
8. Argus, D. F. & Gordon, R. G. *J. geophys. Res.* **95**, 17315–17324 (1990).
9. Smith, D. E. et al. *J. geophys. Res.* **95**, 22013–22041 (1990).
10. DeMets, C., Gordon, R. G., Argus, D. F. & Stein, S. *Geophys. J. int.* **101**, 425–478 (1990).
11. Robbins, J. W., Smith, D. E. & Ma, C. *Crustal Dynamics* (American Geophysical Union, Washington DC, in the press).

Ancestral arthropod

A FOSSIL arthropod from the Silurian (about 420 million years ago) of Australia may represent a transitional form between myriapods and insects (K. J. McNamara and N. H. Trewin, *Palaeontology* **36**, 319–335; 1993). The animal, named *Kalbarria brimmellae*, was about 120 mm long, had a poorly demarcated head, a thoracic region loosely regionated into mono-, diplo- and triplosegments and bearing 11 pairs of legs, and an appendage-free abdomen. *Kalbarria* is a euthycarcinoid, a group of arthropods known only from fossils of Upper Carboniferous and Middle Triassic age. Although a close relationship with insects has been proposed, fully evolved winged insects were in evidence by the Lower Carboniferous. But *Kalbarria* lived 120 million years earlier than the previous earliest-known example of its group, so it is possible that insects evolved from a myriapod-like ancestor via something that looked like a euthycarcinoid.

Strength to strength

IN 1990, the Japanese firm of Kobe Steel Ltd developed a carbon steel wire with a tensile strength of some 5 GPa, well above the previous limit of about 3 GPa. Why this remarkable increase? H. K. D. H. Bhadeshia and H. Harada may have found the answer (*Appl. Surf. Sci.* **67**, 328–333; 1993). Using field ion microscopy, they found that the severe deformation during manufacture produces a very fine structure of dislocation cells in the wire, and also forces much of the carbon in the steel into solid solution. Both effects hamper the movement and growth of dislocations — narrow defect regions in the crystal lattice — in the wire, so that macroscopic yielding is triggered only at higher loads. The wire makes excellent fishing line — so will 'the one that got away' now be 70% larger than before?

Sticks and stones

ELEPHANTS are arguably the most frequent mammalian tool-users after humans and chimpanzees. Just as in primates, tool use is not a single, stereotyped kind of behaviour but an adjunct to a wide range of activities which together reflect the animals' adaptive needs (S. Chevalier-Skolnikoff and J. Liska, *Anim. Behav.* **46**, 201–219; 1993). So whereas small, vulnerable cebid monkeys use tools in aggressive displays, elephants tend to use them to keep cool (squirt mud or water on themselves) or free from parasites (using the trunk to handle sticks or grasses to scratch itches or swat flies). Aggression (brandishing branches or throwing rocks) is less important. The authors speculate that the need for artificial aids to personal hygiene is keenly felt in large, furless tropical land mammals.

■ In last week's *Résumé*, the reference for the final item is *Appl. Phys. Lett.* **63**, 15–17; 1993.

A genetic rainbow of plastids

Jeffrey D. Palmer

MENTION chloroplasts and their genomes, and one thinks of photosynthesis and its associated genes. Land-plant chloroplast DNAs (cpDNAs) are indeed geared almost entirely towards photosynthesis, but work by Reith and Munholland¹ now shows that a red-algal chloroplast genome encodes a surprising number and variety of proteins. Yet despite this genomic diversity, there is increasing reason¹⁻³ to think that all plastids derive from a common, cyanobacterial ancestor.

The view of cpDNA as a genetic engine for photosynthesis was set in the 1980s by the complete sequencing of three land-plant genomes⁴⁻⁶. At least 101 of the 113 products of tobacco cpDNA contribute to photosynthesis and associated chloro-respiration, either directly or indirectly (through gene expression). Studies⁷ on a non-photosynthetic plant suggest that only two to four of the remaining genes are likely to contribute to non-photosynthetic metabolism. The bryophyte *Marchantia polymorpha* contains but a few more plastid genes^{5,8}, and the 'green' alga *Euglena gracilis* contains notably fewer⁹.

In the past several years there has been a stream of reports^{1,10,11} of 'novel' plastid genes in non-green algae, especially *Cyanophora paradoxa* and *Cryptomonas* ϕ . The dam has finally broken with the appearance of the first comprehensive study¹ of a non-green cpDNA, from the red alga *Porphyra purpurea* (see photo).

Reith and Munholland strategically sequenced some 60 per cent of this 188-kilobase genome and found more genes (over 125) than in any of the completely sequenced plastid genomes. This leads to two important conclusions. First, *Porphyra* cpDNA contains roughly twice as

of them) found in *Porphyra* cpDNA greatly increase the known repertoire of chloroplast gene products (ref. 1; Reith and Munholland, personal communication). Half of the genes encode additional translational components and photosynthetic proteins, but the rest are full of surprises. Their products, identified largely by bacterial homologues, include enzymes of intermediary metabolism (nitrogen assimilation, redox reactions and biosynthesis of amino acids, fatty acids, phycobilins, carotenoids and chlorophyll); agents of protein transport (*secA* and *secY*), assembly (the chaperonins HSP70 and HSP60) and processing (*clpB*); and factors involved in DNA replication (*dnaB*) and transcriptional regulation (members of the *OmpR*, *LysR*, *EnvZ* and *crp* families). So plastid gene diversity in *Porphyra* begins to mirror, at least qualitatively, the physiological and biochemical diversity of plastids.

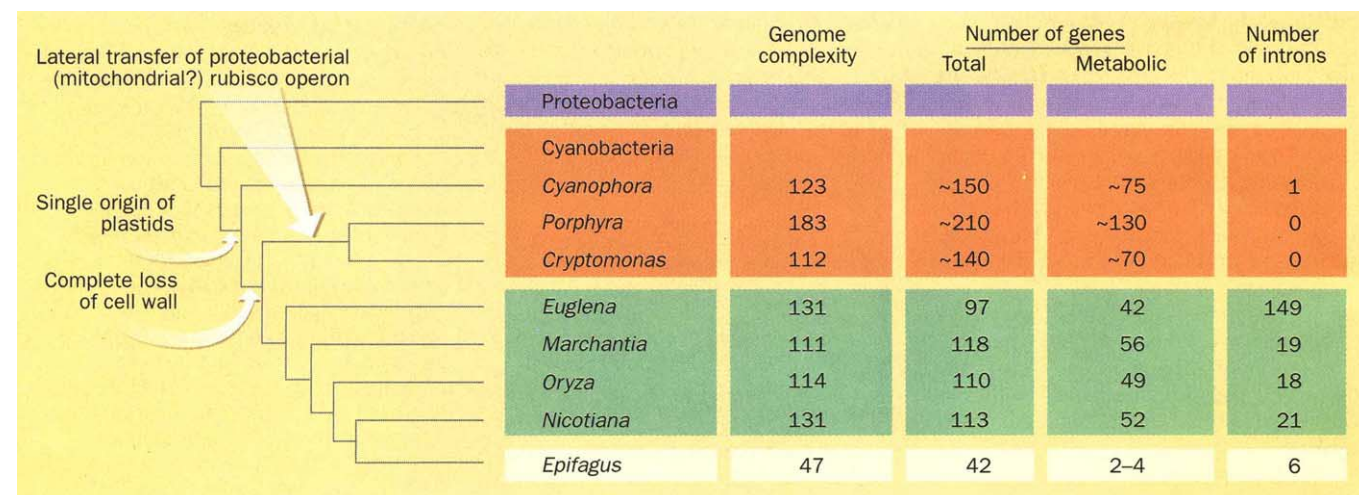
Overall, the numbers of all genes and of metabolic genes (the *raison d'être* for maintaining an expressed genome) vary about two- and threefold, respectively, for photosynthetic plastids, and if plastids of non-photosynthetic eukaryotes are included the ranges increase to roughly fivefold and 40-fold (see table). Assuming plastid monophyly (see below), this implies large, lineage-specific differences in the amount of post-endosymbiotic loss of genes from the plastid and concomitant (in some cases) relocation of function to the nucleus, either by direct gene transfer or indirect gene substitution¹². Yet these differences in chloroplast gene content are still dwarfed by the nuclear contribution to

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Porphyra purpurea — most primitive plastid genome known.

many genes as land-plant genomes. Although many of the hitherto unknown plastid genes in *Porphyra* are also found in *Cyanophora*¹¹ and *Cryptomonas*¹⁰, the latter genomes are much smaller in size and gene content (see table), making *Porphyra* the champion and, in this respect, the most primitive¹ plastid genome known.

Second, the novel genes (more than 60



Tentative phylogeny of plastids onto which are mapped data on plastid gene and intron content (see refs 1-14). Plastids are shown whose genomes are either completely (the bottom five) or substantially (the top three) sequenced, so land plants are over-represented. The numbers of 'metabolic' genes include unidentified open reading frames and thus may be overestimates. *Cryptomonas* and *Euglena* are thought to have acquired their plastids through secondary symbiosis within eukaryotes^{10,13}. White, non-photosynthetic plastids without pigments; green, plastids with chlorophyll *b*; red, plastids and bacteria with phycobilins; purple, proteobacteria.

plastid protein content; even in *Porphyra*, some 80 per cent of the 1,000 or so different plastid proteins must be nuclear-encoded. Thus, the bulk of plastid gene loss and relocation probably occurred soon after endosymbiosis, before the rise of the main algal groups.

Why do gene content and diversity vary so dramatically among cpDNAs (and also among mitochondrial DNAs¹³)? In my view, it probably doesn't matter whether a plastid has a genome at all, just as long as it gets all the proteins it needs, in the right doses, and at the right times. Accordingly, one should be able to rid some nearly extinct plastid (for example *Epifagus*⁷, see table) of its genome by the deliberate re-engineering of its remaining metabolic genes to the nucleus. The present diversity of gene contents may largely reflect chance accidents of evolution combined with historical contingency. That chloroplast gene content, even in *Porphyra*, is so biased towards thylakoid and translational proteins is probably a function of mechanistic factors that impede gene relocation to the nucleus rather than selection 'favouring' a certain division of genetic labour. Thylakoid proteins should be most difficult to relocate, for these lack substitutable homologues in the nucleus and are poor candidates for successful gene transfer owing to their hydrophobicity and constrained evolution (these are part of multisubunit complexes and also change very slowly in sequence). Conversely, genes for soluble enzymes (of which even *Porphyra* cpDNA contains few) should be most readily relocated, perhaps largely through gene substitution¹².

Reith and Munholland¹ found that the *Porphyra* genome also contains two notable structural features. First, it lacks introns. Because only two introns, one of cyanobacterial origin, have been found in all non-green plastid genomes^{8,12,14}, this reinforces the idea^{9,12} that the many, lineage-specific introns in green plastid genomes (see table) are of post-endosymbiotic origin. Second, *Porphyra* cpDNA contains two distinctive gene clusters that are uniquely shared with land

plants and *Cyanophora* relative to cyanobacteria. Interpretation¹ of these clusters as evidence for a monophyletic origin of plastids is clouded by the obviously reductive, perhaps convergently so, evolution of chloroplast gene content. Yet these features are congruent with phylogenies for an increasing number of chloroplast genes that point to a monophyletic, cyanobacterial origin of all plastid lineages (see table)^{2,13}.

The case for plastid monophyly must, however, reckon with two striking anomalies. The first, a seven amino-acid gap in *psbA* shared by green chloroplasts and prochlorophytes, is now best explained as simple homoplasy (parallel deletion in each lineage)^{2,13}. The second anomaly has much deeper implications. Both rubisco genes (*rbcL* and *rbcS*) are biphyetic: those of red algae, cryptomonads and chromophytes cluster strongly with

certain proteobacteria, whereas those of green plastids and *Cyanophora* are of cyanobacterial affinity^{2,3}. This conflict with the apparent monophyly of all other plastid genes has been explained by invoking either horizontal transfer of a proteobacterial rubisco operon (see table) or else differential retention of two rubisco operons present in the cyanobacterial ancestor of plastids^{2,3}. So despite the evidence for a monophyletic origin of plastids, it is clear that their post-endosymbiotic history has been rich in unexpected events of differential gene relocation to the nucleus and lateral or paralogous evolution, not to mention long-suspected cases of secondary symbiosis within eukaryotes^{10,13}. □

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CELLULAR CALCIUM

A mysterious new influx factor?

David E. Clapham

IMAGINE you are standing under a large tarpaulin filled with water. You occasionally run your hand along the undersurface and allow a few drops of water to squeeze through small holes in the tarpaulin. This is the situation at the plasma membrane of a cell, where the concentration of calcium outside the cell is enormous — roughly 10,000 times higher than that inside the cell. Occasionally a cellular 'hand' lets in a dribble of calcium, refilling the cell's stores. One of the most intriguing mysteries of cell biology is how emptied intracellular calcium stores signal to the plasma membrane to let in more calcium. On page 809 of this issue¹, Randriamampita and Tsien propose that a novel calcium influx factor, CIF, gates this entry mechanism. A second report by Parekh *et al.*² on page 814 supports key aspects of their proposal.

Numerous receptors on the plasma membrane, such as the serotonin 5HT_{1C} receptor, initiate a signal transduction cascade (see figure on next page) resulting in the rapid release of calcium stored in the endoplasmic reticulum (ER). Calcium flooding into the cytoplasm is a mediator in a panoply of cellular responses, ranging from contraction of smooth muscle to exocytosis in secretory cells³. Once released, however, calcium is pumped both out of the cell and back into the ER by Ca-ATPase pumps. To replenish the ER calcium, specific ion channels in the plasma membrane open to allow a small amount of extracellular calcium to enter the cytoplasm and eventually to be pumped into the calcium store. The stimulated calcium entry may

also serve to activate effectors in a different spatial domain from that of ER-released calcium. The mysterious mechanism by which the depleted ER signals the cell membrane to let in more calcium is called the capacitive entry pathway⁴.

Although many proteins that might refill calcium stores have been identified, such as voltage-activated calcium channels and exchangers, the cell uses other specialized proteins. Recently, ion channels responsible for one current have been characterized and the current has been called the calcium release-activated current (*I_{CRAC}*; refs 5, 6). This current has several unique properties including an extremely low single-channel conductance (about 20 femtosiemens), high calcium-selectivity (even when presented with other divalent cations), and little or no voltage-dependence for channel opening. As the equilibrium potential for calcium is about +150 millivolts, the more hyperpolarized the cell, the more calcium is brought into it. Although this calcium channel is the only one so far to be linked to depletion of intracellular calcium stores, other receptor-activated calcium channels have been described⁷.

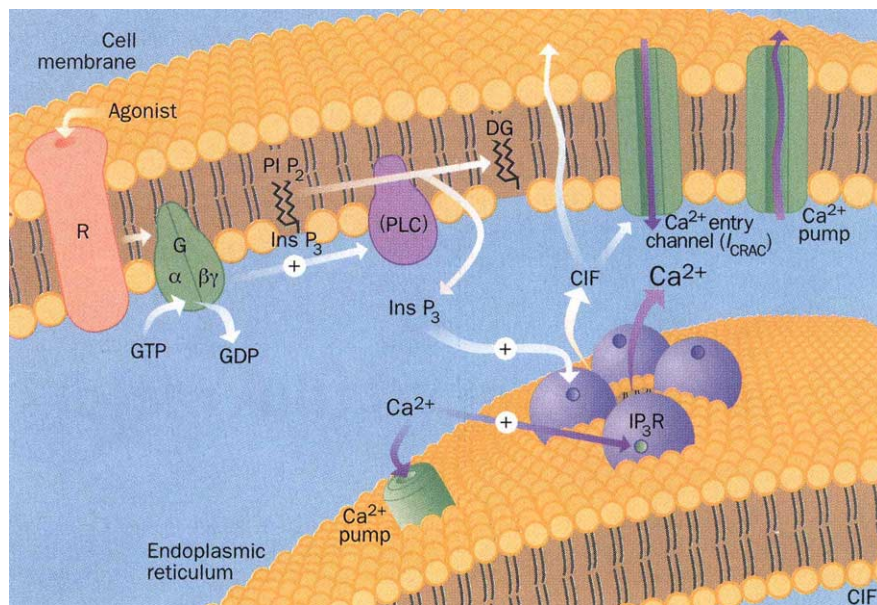
Now, Randriamampita and Tsien have partially characterized a small (*M_r* less than 500), anionic, phosphorylated compound that activates calcium entry when applied to cells. This factor, CIF, is probably not a peptide and appears to have hydroxyls, or a hydroxyl and amino group, on adjacent carbons. It is generated by cells whose calcium stores have been depleted by any one of four ex-

1. Reith, M. & Munholland, J. *Pl. Cell* **5**, 465–475 (1993).
2. Morden, C. W. *et al.* *Biosystems* **28**, 75–90 (1992).
3. Martin, W., Somerville, C. C. & Loiseau-de Goër, S. *J. molec. Evol.* **35**, 385–404 (1992).
4. Shinzaki, K. *et al.* *EMBO J.* **5**, 2043–2049 (1986).
5. Ohyama, K. *et al.* *Nature* **322**, 572–574 (1986).
6. Hiratsuka, J. *et al.* *Molec. gen. Genet.* **217**, 185 (1989).
7. Wolfe, K. H., Morden, C. W. & Palmer, J. D. *Proc. natn. Acad. Sci. U.S.A.* **89**, 10648–10652 (1992).
8. Wolfe, K. H., Morden, C. W. & Palmer, J. D. *Curr. Opin. Genet. Devel.* **1**, 523–529 (1991).
9. Hallick, R. B. *et al.* *Nucleic Acids Res.* **21**, 3537–3544 (1993).
10. Douglas, S. E. *Biosystems* **28**, 57–68 (1992).
11. Löffelhardt, W. & Bohnert, H. J. in *Molecular Biology of the Cyanobacteria* (ed. Bryant, D. A.) (Kluwer, Dordrecht, in the press).
12. Palmer, J. D. in *Molecular Biology of Plastids* (eds Bogorad, L. & Vasil, I. K.) 5–53 (Academic, San Diego, 1991).
13. Gray, M. W. *Int. Rev. Cytol.* **141**, 233–357 (1992).
14. Bernard, C. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **89**, 9564–9568 (1992).

perimental manoeuvres. CIF moves from organelles to cytoplasm, and even across the cell membrane into the supernatant, where it can stimulate adjacent cells. A clever set of cross-desensitization experiments has determined that CIF is not a recognized conventional second messenger.

Independent electrophysiological experiments seem to bear out some of the

there is early evidence from I_{CRAC} (ref. 9) and calcium measurements¹⁰ which hints at the involvement of small G proteins as the coupling messenger. I_{CRAC} is not the only channel admitting calcium at hyperpolarized potential after calcium release⁷. Finally, other proposed direct coupling schemes between the ER inositol-trisphosphate receptor and the plasma-membrane repletion channels^{11,12} have



CIF, or calcium influx factor, is apparently released from intracellular organelles when calcium stores are depleted. CIF opens calcium influx pathways, probably ion channels, to admit calcium back into the cytoplasm where Ca-ATPase transporters then pump it back into the cell's stores. In many cells, calcium is released from the endoplasmic reticulum (ER) via activation of the inositol trisphosphate (InsP_3) receptor. Many GTP-binding protein receptors are coupled to phospholipase C to break down phosphatidylinositol bis phosphate (PIP_2) into diacylglycerol (DG) and InsP_3 . InsP_3 opens a receptor/channel on the ER membrane to allow calcium to flow out of the ER into the cytoplasm.

main characteristics of the mobile factor. Using *Xenopus laevis* oocytes expressing 5HT_{1C} receptors, Parekh and colleagues show that an I_{CRAC} -like current is enhanced by okadaic acid, a serine/threonine phosphatase inhibitor (class 1A, 2A). Furthermore, when a piece of plasma membrane is ripped from the cell the intrinsic mechanism runs down, but if the excised patch is crammed back into the cytoplasm at a different cellular location, the patch may once again respond. This indicates that a diffusible phosphorylated calcium influx factor is inactivated by intrinsic phosphatases, supporting key aspects of CIF.

Is CIF the long-sought factor? Are there alternative mechanisms? The two reports published here will almost certainly not settle the issue. First, CIF has been only partially characterized and a crucial experiment, the activation of I_{CRAC} by CIF in an electrophysiological assay, has not yet been done. Also, after longer incubation periods in okadaic acid, refilling of stores is apparently uncoupled rather than enhanced⁸. Furthermore,

yet to be tested experimentally. Only time will tell if CIF will join the ranks of unexpected, new second messengers such as nitric oxide and cyclic ADP ribose. In the meantime, these results will provide new tools and stimulate further experiments to elucidate one of the cell's most important control mechanisms. □

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1. Randriamampita, C. & Tsien, R. Y. *Nature* **364**, 809–814 (1993).
2. Parekh, A. B., Terlau, H. & Stühmer, W. *Nature* **364**, 814–818 (1993).
3. Berridge, M. J. *Nature* **361**, 315–325 (1993).
4. Putney, J. W. Jr *Cell Calcium* **11**, 611–624 (1990).
5. Hoth, M. & Penner, R. *Nature* **355**, 353–356 (1992).
6. Zweifach, A. & Lewis, R. S. *Proc. natn. Acad. Sci. U.S.A.* **90**, 6295–6299 (1993).
7. Neher, E. *Nature* **355**, 298–299 (1992).
8. Berlin, R. D. & Preston, S. F. *Cell Calcium* **14**, 379–386 (1993).
9. Fasolato, C., Hoth, M. & Penner, R. *J. biol. Chem.* (in the press).
10. Bird, G. St. J. & Putney, J. J. *J. biol. Chem.* (in the press).
11. Berridge, M. J. *J. biol. Chem.* **265**, 9583–9586 (1990).
12. Irvine, R. F. *FASEB J.* **6**, 3085–3091 (1992).

How to be loved

ONE of the saddest facts of human life is that the demand for love greatly exceeds the supply. Hence such diverse phenomena as agony columns, romantic novels, philosophy, creative art, domestic pets, and crime. The artist, it is said, is belatedly seeking the love and approval he did not get in infancy. And it is the children of broken homes and fostering agencies who, starved of early love, develop into thugs and vandals.

In this connection, Daedalus recalls the strange psychiatric syndrome of free-floating anxiety. The sufferer isn't anxious *about* anything; he's just anxious. Similarly, many teachers encourage their pupils to feel free-floating self-esteem, despite having nothing much to be esteemed for. And we all envy the free-floating celebrity enjoyed by those popular figures who aren't celebrated for anything: they're just celebrities. So, says Daedalus, what we need is a way of inoculating children with free-floating belovedness — the sense of being loved or having been loved, not by anyone in particular, just loved in the abstract.

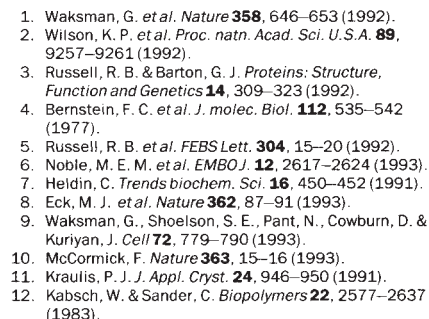
This apparent spiritual problem may in fact be biochemical. Simple anaesthetics can abolish from the brain its magical property of consciousness. Anxiolytics and tranquillizers can conquer the dark forces of misery and loosen the grip of obsession. So DREADCO's biochemists are now seeking the molecular basis of belovedness. They are studying the brains of young hooligans knifed or shot by their fellows, and comparing them with those of happy and stable citizens who, less deservedly, have also met with fatal accidents. Somewhere in this complex comparison, they hope to spot the key enzyme level or neurotransmitter-ratio which, once properly set, gives its owner the ontological security of feeling that he has been loved.

DREADCO's 'Be-Loved'® will be loaded into the child's own T-cells. They will then be injected back into him, to carry the drug through the blood-brain barrier. It will then trip the vital biochemical switch normally tripped by growing up in a secure family. The resulting permanent sense of belovedness and self-worth will be an enduring inoculation against crime. It should be given pre-emptively: ideally at parental separation, and certainly no later than first conviction. Be-Loved will be vastly expensive — it will push the state of the art to develop. But it will easily be paid for by the wholesale closure of police and social services departments. The entire underclass of their clientele will be transformed into stable, hard-working, complacent, artistically untalented citizens.

David Jones

Both SH2 and BirA bind phosphate. SH2 binds phosphotyrosine-containing peptides, whereas BirA catalyses the

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Scientific Correspondence is a relatively informal section of *Nature* in which matters of general scientific interest, not necessarily those arising from papers appearing in *Nature*, are published. Because there is space to print only a small proportion of the letters received, priority is usually given according to general interest and topicality, to contributions of fewer than 500 words, and to contributions using simple language.

No ice-sheet collapse

SIR — Blankenship *et al.*¹ present intriguing evidence for a subglacial volcano that supports the characterization of central West Antarctica as a rift zone². I believe it highly unlikely that a volcano (even many volcanoes), or the properties of the lithosphere that they may represent, would make the ice sheet vulnerable to collapse.

The possibility of unstable collapse is important because it carries the implication of a serious rise in sea level. Blankenship *et al.* write that a retreat of the downstream termini of the ice streams (grounding line) to a proposed boundary between hot and cold lithosphere, marked approximately by the volcano, "would almost certainly represent an unstable situation", because the buffering ice streams would be unable to migrate upstream past that boundary. I do not dispute the likely instability of that situation if it were to arise, but the authors place that boundary about two-thirds of the way from the present grounding line to the centre of the ice sheet. A grounding-line retreat of such magnitude would probably mean that an instability had already been triggered. Further, it could hardly occur without induced retreat of adjacent drainage systems through the downdraw process³, which would in turn imply that much of the West Antarctic ice sheet had already spread into the ocean. With grounding lines then deep into the Byrd subglacial basin, instability would be virtually assured with or without migrated ice streams. Thus the authors' proposed collapse mechanism would come into play so late in West Antarctic ice-sheet shrinkage as to be largely irrelevant. The important point with regard to ice-sheet collapse is the stability of the ice stream as it now exists, which has nothing to do with lithospheric boundaries.

More fundamentally, I question the conclusion of Blankenship *et al.* that "elevated geothermal flux provides an important control on the dynamics of the [West Antarctic ice sheet]". Certainly, elevated heat flow would enhance melting from the underside of the ice sheet. But the coexistence of enhanced melt and ice streams is not evidence of cause and effect. Ice streams and other fast-moving glaciers are a common phenomenon in ice sheets past⁴ and present⁵, including those on presumably cold Precambrian shields in East Antarctica and Canada. Few, if any, are associated with volcanism — ample water is provided by the heat of glacial sliding⁶, so elevated heat flux is not generally needed for fast glacier flow. Is there reason, then, to suppose that high heat flow is an important control on West

Antarctic ice streams? The authors cite no supporting evidence, nor has any such evidence been reported in extensive study of those ice streams over the past decade (see ref. 7 for a summary).

In summary, although the geophysical evidence for volcanism provides interesting support for active rifting in West Antarctica, it should not be taken as threatening an imminent collapse of the West Antarctic ice sheet, contrary to some press reports that have appeared.

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- Blankenship, D. D. *et al.* *Nature* **361**, 526–529 (1993).
- LeMasurier, W. E. *Geol. Soc. Am. Abstr. Progr.* **10**, 443 (1978).
- Stuiver, M., Denton, G. H., Hughes, T. J. & Fastook, J. L. in *The Last Great Ice Sheets* (eds Denton, G. H. & Hughes, T.) 319–436 (Wiley-Interscience, 1981).
- Clark, P. U. *EOS* **74**, 310 (1993).
- Bentley, C. R. *J. geophys. Res.* **92**, 8843–8857 (1987).
- Lingle, C. S. & Brown, T. J. in *Dynamics of the West Antarctic Ice Sheet* (eds Van der Veen, C. J. & Oerlemans, J.) 249–285 (Reidel, Dordrecht, 1987).
- Alley, R. B. & Whillans, I. M. *Science* **254**, 959–963 (1991).

Sequence data as evidence

SIR — The debate^{1,2} concerning the proposed transmission of HIV to five patients of a dentist suffering from AIDS highlights the difficulties in establishing networks of HIV transmission from the analysis of viral nucleotide sequence data. The details of that particular case notwithstanding, we believe that there are issues that need to be addressed before HIV sequence data alone can be used to resolve such questions.

The first issue is the choice of region of the HIV genome which provides the best estimate of relatedness. Ou *et al.*¹, in their analysis of the Florida dentist case, used sequences from the C2–V3 region of the *env* gene. This region was considered to be suitable for tracing transmission networks because of the extensive variability in sequence and the large amount of homologous data already available. However, identical V3 loop sequences have been observed to evolve in epidemiologically unrelated patients³ (convergent evolution) and individuals in the late stages of the disease often possess more diverse sequences than those obtained from early in infection^{4,5}. Such complex evolutionary patterns will hinder accurate determination of the probabilities of transmission.

In another investigation of HIV transmission to a Swedish rape victim, Albert *et al.*⁶ analysed a region of the more conserved *pol* gene. Although shared substitutions in this region may be given more weight because they are relatively rare, it is likely that there may not always be sufficient variability to resolve particularly close relationships. Finally,

we have used a region of the *gag* gene, encompassing part of the p17 protein, to determine whether an HIV infected surgeon had transmitted the virus to a patient⁷. This region has an intermediate level of variability and such sequences were found to duplicate known pathways of transmission between individuals in a detailed analysis of HIV evolution in Edinburgh (E. C. H. *et al.*, manuscript in preparation).

A second issue concerns the method of analysis of the sequence data. The most obvious approach involves the inference of the phylogenetic relationships of the sequences in question. It is possible, however, that HIV evolves in ways incompatible with the assumptions made by many methods of phylogenetic analysis. An example is again provided by the observation of convergent evolution in the V3 loop^{3,8}. Such reservations also apply to the 'signature' sequence analysis undertaken by Ou *et al.*¹ because, like cladistic methods of phylogenetic inference, it implicitly assumes the independent and divergent evolution of the relevant amino-acid residues. Furthermore, in this type of investigation it is crucial that an estimate of uncertainty be assigned to tree topologies, such as that provided by bootstrap resampling. An alternative method is based on the comparison of the log likelihoods of tree topologies which depict competing transmission networks⁷. Whatever the approach taken, it is important to recognize that the probabilities which can be attached to different phylogenies are orders of magnitude lower than those which are used in DNA fingerprinting based on VNTR sequences.

We believe that close attention to the points raised here, together with more detailed studies of the nature of HIV sequence evolution within communities, are required before HIV nucleotide sequence data can be regarded as evidence which can be used uncorroborated in a legal context.

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- Ou, C. Y. *et al.* *Science* **256**, 1165–1171 (1992).
- DeBry, R. W. *et al.* *Nature* **361**, 291 (1993).
- Zhang, L. Q. *et al.* *J. Virol.* **67**, 3345–3356 (1993).
- Chesebro, B., Wehrly, K., Nishio, J. & Perryman, S. *J. Virol.* **66**, 6547–6554 (1992).
- Schuitmaker, H. *et al.* *J. Virol.* **66**, 1354–1360 (1992).
- Albert, J., Wahlberg, J. & Uhlen, M. *Nature* **361**, 595–596 (1993).
- Holmes, E. C. *et al.* *J. infect. Dis.* **167**, 1411–1414 (1993).
- Holmes, E. C. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **89**, 4835–4839 (1992).

Impoverished minds

Stuart Sutherland

B. F. Skinner: A Life. By Daniel W. Bjork. *Basic Books*: 1993. Pp. 282. \$25. To be published in the United Kingdom by HarperCollins in November, £16.99.

To become a guru, it is necessary to shout louder and longer than anyone else, but the message itself must either be so unintelligible that everyone thinks it important because they cannot understand it (compare, for example, Michel Foucault and some contemporary neuroscientists and physicists) or be so simplistic that everyone can congratulate themselves on understanding it to the full.

B. F. Skinner chose the latter method. He argued that all behaviour could be explained in terms of genetic factors and the history of reinforcement to which the organism has been exposed, a belief that is patently absurd: people learn about their environment when no reinforcement is provided. Again, it has been repeatedly shown that if people are rewarded for performing a task they find intrinsically pleasurable, they do it less not more. To overcome these problems, Skinner postulated the existence of internal rewards such as the satisfaction of curiosity or the thrill of fear, but this assumption means that behaviour is *not* controlled by external events and cannot be predicted. Nor can we control a given person's internal satisfactions. In fact the postulation of internal rewards flies in the face of one of Skinner's major tenets, namely, that there are no internal events: at times he even denied the existence of consciousness. Again, he did not appreciate the difficulty of defining what constitutes a stimulus or what is the action that is reinforced: Noam Chomsky commented that if you enjoy reading a book, you should immediately read it again.

Skinner's research was almost as empty as his theorizing, but he made three (and only three) contributions to psychology: curiously none of them is mentioned by Daniel Bjork. Skinner discovered that if a response is rewarded randomly on only some of the occasions on which it is emitted, the organism gives more responses after reinforcement is withdrawn than if all previous responses had been reinforced. (This discovery had in fact been made many years earlier by G. C. Grindley at Cambridge, England, but his work was ignored.) Second, Skinner pointed out that punishment is a less effective method of controlling behaviour than reward, because punishment merely stops the organism doing something, leaving it free to substitute any other form of behaviour. Third, Skinner was the first person to distinguish clearly between Pavlovian classical conditioning

and the operant conditioning on which he worked: unfortunately he ignored this distinction in his later years.

It is true that Skinner saw the desirability of recording behaviour automatically in order to increase accuracy (and possibly to allow the experimenter to slip out for a cup of tea in the middle of the experiment). To this end, using his con-

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Behaviourally flawed: Skinner (1904–1990).

siderable practical skill he invented the Skinner box in which the organism can effectively only make one response — pressing a key or pecking a panel: the device has been well described as a bloodless method of decerebrating both the animal and the experimenter. By his oversimplification, Skinner held back the progress of animal — and to a lesser extent human — psychology for a generation. The workings of the mind are extremely rich and by denying the mind's existence Skinner prevented his disciples from studying it. If Skinner had been right, psychology would be a dull subject indeed. Much to his dismay, with the advent of cognitive psychology, the richness of the workings of the mind and the possibility of studying them scientifically were recognized long before his death.

B. F. Skinner: A Life gives an impoverished and completely uncritical account of Skinner's scientific work. It is better on his attempts to influence the public, which started with his novel *Walden Two* depicting a Utopia in which everyone's behaviour was completely controlled by well thought out reinforcements supplied

for the good of everyone. Apart from the fact that, as we have seen, it is quite impossible to control people in this way, Skinner failed to ask what the ends of a Utopian society should be or who should decide those ends. He also invented the "Heir Conditioner" (a good name), which was an air-conditioned box in which a baby could live with freedom of movement and with a minimum of soiling from its own excreta, and he was one of the first to devise a teaching machine. None of these projects came off. *Walden Two* caused an uproar: although many defended Skinner's Utopia, others thought it resembled Fascism. A few communities based on *Walden Two* were established, but Bjork does not tell us what became of them. Skinner's attempts to market his Heir Conditioner and teaching machine were also a failure, partly because he appears to have had no business sense.

As to Skinner's life, Bjork records little of interest that is not to be found in Skinner's own three-volume autobiography. It is admittedly hard to paint a vivid picture of a man who denies having feelings or emotions, but Bjork completely fails at this task. What was Skinner really like? Ambitious, certainly; naive, definitely; kind to his family and associates, probably; genuinely interested in the welfare of mankind, unlikely. Moreover, Bjork omits much that is of interest. He does not mention Skinner's visits to Esalen, the Californian centre of humanistic psychology, where the psychotherapists Frederick Perls, A. H. Maslow and Carl Rogers strutted around in the nude among their army of (mainly female) disciples. Their views could hardly have been more antithetic to Skinner's own rejection of the self, for they believed not merely in the self but in "the true self" whose discovery frees one from mental disorder and anxiety and produces perfect happiness. What, one wonders, did the four of them discuss in the bizarre encounter groups that were one of Esalen's main features? Again, Bjork makes only one brief reference to Skinner's habit of providing himself with positive reinforcement by his notorious and obsessive womanizing: Skinner was a rummer character than Bjork allows.

The last time I saw Skinner I offered to take him and his wife up Magdalen Tower in Oxford, England. On seeing the narrow staircase, his wife declined because she had claustrophobia. When Skinner and I reached the top, he refused to go near the edge because he had acrophobia. Despite Skinner's faith in behaviour therapy, these examples seem to indicate that not all behaviour can be changed by a system of rewards and punishments. □

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Nothing doing

Keith Stewart Thomson

Science as a Way of Knowing: The Foundations of Modern Biology. By John A. Moore. Harvard University Press: 1993. Pp. 530. \$29.95, £23.95.

THIS wonderfully readable book has two main components: a blow-by-blow account of some key advances in biology from, roughly, Aristotle to Watson and Crick, and an explanation of the line of reasoning behind scientific discovery in the natural sciences (some Earth history is included). Moore defines the core of biology as the phenomena of self-replication, diversity and change over time. He is concerned therefore with understanding the intertwining strands of genetics, development and evolution.

In time-honoured fashion, the book begins with the Greeks and proceeds steadily to Galen and then Francis Bacon. Most of this first part is a bit tame, particularly because several nuggets of ancient thought are omitted only to turn up in later sections (in a discussion of pangenesis on page 235, for example, we learn that Hippocrates defined the scientific method). Moore gives short shrift to Aristotle's considerations of cause, leaving him as the founder of descriptive science. As a result, not until page 93 do we move from the more vague phrasology of "understanding nature" to an uncompromising search for "fundamental causes or the association of seemingly

unrelated events". By this time we are well into Baconian induction and deduction. Similarly, it is not until page 405, in the discussion of Karl Ernst von Baer, that we learn that "scientists assume that there are general rules that apply to natural phenomena" (as if Aristotle never thought about this).

Although such omissions are irritating in a book that otherwise marches from point to point, the general effect is a clever interweaving of ideas that continues to develop right to the end. Moore's writing is marvellously accessible, and scientists and nonscientists alike will enjoy his frequent lighthearted asides, such as (on the Scholastics): "This was the Abelard who had an affair with the beautiful and loving Heloise. Her father felt strongly about that and had Abelard castrated to cool his ardor. It did."

Moore's treatments of genetics and development are superbly clear and read like a mystery story in which the clues are (sometimes too neatly) unravelled one by one, although the informed reader may well wonder, first, what everyone else was doing and, second, whether anything worth knowing happened after Watson and Crick or Hans Spemann. To be sure, in physical science, water always consists of hydrogen and oxygen (p. 136), but biological investigations are never-ending; one can never be sure that we know whodunit, because both 'who' and 'it' keep shifting. Particularly lacking here is any treatment of the emergence of molecular biology as the common language of biology, rather than simply the most reductionist end of genetics. Also

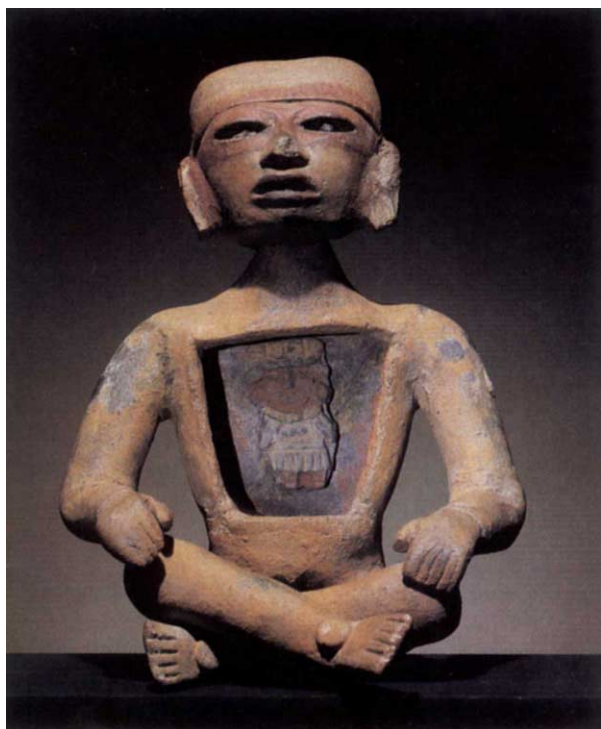
lacking, simply because Moore has chosen to write a different, more Polyanna-ish sort of book, is any hint of what the day-to-day practice of science is like — the false starts, mindless repetition, faddishness and clannishness. This book is about science as knowing, not science as doing.

Moore's purely internalist history of biology unfolds in a philosophical-political-socioeconomic vacuum except for the standard posing of religion as the enemy of free inquiry. I was struck, however, by the statement that whereas religion is subject to dogma "forcefully promoted by priests and rulers, who may be greatly rewarded by so doing", science is free from these ills because of the nature of scientific reasoning. Oh really!

Moore's expository method of carefully tracing the logic of the usual process of advance through data accumulation, induction, hypothesis, deduction and testing, is, surprisingly, weakest where he applies it first: to organic evolution and the science of Charles Darwin. In Chapter 8 ("Testing Darwin's Hypotheses"), Moore introduces confusion by forcing the central concepts of evolution and the theory of natural selection into an artificial framework. One can see what he is trying to do — as Darwin himself stated: "[my] line of argument . . . is to establish a point as a probability by deduction and to apply it as hypotheses to other points to see whether it will solve them". Thus, Moore writes: "If all today's species are descendants of a few original forms" then "there should have been connecting forms between the major groups". The fossil record then becomes a test of this deduction. But the deduction that "the species that lived in the remote past must be different from the species alive today" does not flow from the hypothesis but is a premise of the hypothesis. The same is true for the deductions that "There must be variation among organisms. . ." and that "Natural selection can only be operative if more offspring are born than survive". The deduction that "If the members of a taxonomic group . . . share a common ancestry, that fact should be reflected in their structure" suffers from a different problem, the old one of tautology. Taxonomic groups, ever since Aristotle, are defined by common structure in the first place.

These difficulties apart, this volume is a worthy addition to the literature on the history of biology. It explains the foundations of evolution, genetics and development and the logic behind scientific enquiry with a clarity that will put most writers of elementary textbooks to shame. It both demystifies science and exalts it. □

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TEOTIHUACAN figurines such as this one, which dates from between AD 650 and 750, are thought either to express the *nahual* or "double that we all possess and into which we may transform ourselves" or to represent a goddess of the Earth who receives and protects dead warriors. The picture is one of over 250 included in *Teotihuacan: Art from the City of the Gods* edited by K. Berrin and E. Pasztory. The volume is published in conjunction with an exhibition, organized by the Fine Arts Museums of San Francisco, that focuses on the art of this ancient civilization. Thames and Hudson, £28.

Case of curiosities

Brian Pippard

And Yet It Moves: Strange Systems and Subtle Questions in Physics. By Mark P. Silverman. Cambridge University Press: 1993. Pp. 266. £35, \$49.95 (hbk); £12.95, \$24.95 (pbk).

THIRTY years of living with physics have not dulled Mark Silverman's capacity to marvel at the incomprehensible weirdness of the world it describes, and when he finds yet another tantalizing example his instinct is to devise an experiment to make sure it is real. The result is a personal case-history, a series of investigations that a more stolid physicist (and there are many) might dismiss as wholly unnecessary; for if one accepts the standard formulation of quantum mechanics, its paradoxical consequences are inescapable and need no confirmation. Take the Aharonov-Bohm effect — the prediction that the interference pattern produced by splitting and recombining an electron beam may be shifted if magnetic flux threads through the space between the split beams, even though no magnetic field is allowed to reach the electrons themselves. If the effect were found not to occur, the whole structure of quantum mechanics would be undermined, but it was reasonably well verified long ago and I don't suppose Silverman expected to find otherwise — but he had to be quite sure, and used the resources of modern electron microscopy to give an utterly convincing demonstration. Good for him.

This is only the preliminary to more complicated and taxing tests of old and new mysteries. Einstein-Podolsky-Rosen, Hanbury-Brown and Twiss, the Berry phase, the fifth force, Maxwell's demon and many others find their way into a wide-ranging and sometimes not fully articulated argument — Silverman cannot resist throwing in any little thing that fascinates him, even if it is only marginally relevant. Once this is recognized it is seen as part of the fun; and this is a book that is meant to be fun — it's written for those who haven't yet grown out of enjoying intellectual exercise.

Take warning, however; it is not easy reading. The absence of mathematics is no guarantee of a smooth ride — on the contrary, at this level of physics, mathematics is a positive aid, not an encumbrance. The words therefore have to be read very carefully, or the point of the argument slips away. Even with careful reading, the point sometimes eludes the grasp; for example, the discussion about reflection of polarized light by optically active materials is unconvincing because the alternative theories are not

presented fully enough for one to understand what has been discovered by means of a very delicate measurement.

We have here a small book that could have been a much larger book if every topic had been given the detailed attention it deserves. But if it excites curiosity and leads the reader to think hard and to follow up the references for only one or two points, it will have done a useful job. The basic commodity the author offers for sale is his enthusiasm. He wants to share the adventure of tackling the deep intricacies of the physical world with as many others as possible. And it is greatly to his credit as a teacher that he offers no illusory short cuts. □

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Getting down

Simon Klemperer

A Continent Revealed: The European Geotraverse. Edited by Derek Blundell, Roy Freeman and Stephan Mueller. Cambridge University Press: 1992. Pp. 275. £35, \$79.95 (boxed set); £15.95, \$34.95 (paperback book only).

NEWTON claimed that he had seen a little further because he stood on the shoulders of giants. In the Earth sciences, still in equal parts descriptive and analytical, the giants' shoulders on which we stand today are not just prior scientific insights but also the data compilations that beget these insights. One such giant data-collection project has been the European Geotraverse (EGT), a seven-year international study of the lithosphere of the European continent, the results of which are summarized and synthesized in *A Continent Revealed*. But this package is more than a scientific achievement. It is also a publishing *tour de force*, being part textbook, part research monograph, part geoscientific atlas and part digital dataset. It will be read and used in all these ways.

The EGT is one of the best examples of Earth-science collaboration in Europe. From 1983 to 1990, scientists from 14 countries contributed to a series of experiments, particularly the collection of seismic data, to illuminate the three-dimensional structure of a swath from northern Scandinavia to central Tunisia, a palimpsest of 3,500 million years of geological history. The territory studied is 4,600 km long (north to south), 250 km wide and, crucially to the philosophy of the EGT though sometimes only nominally in terms of achievement, 450 km deep. This emphasis on extending surface (and too often superficial) knowledge to

depth is emblematic of one of the great achievements of modern Earth science. Only in the past three decades has it been possible — and only very recently routine — to probe significantly into the third dimension with precise seismic experiments. The EGT went beyond the scope of most national programmes to encompass nonseismic geophysics and geological mapping as well. Elsewhere in the world, only the Canadian Lithoprobe programme has obtained comparable interdisciplinary results.

The detailed scientific results of these experiments have already been published in eight special issues of the journal *Tectonophysics*, the proceedings of six workshops, and two dozen theses. What makes *A Continent Revealed* special is the editors' achievement in summarizing a huge amount of primary scientific literature at a level accessible to an advanced undergraduate, and successfully melding the contributions of an additional dozen authors into a single narrative. But the main paperback book is not only a collection of facts; the authors are happily not afraid to attempt answers, however sketchy, to unsolved problems. This part of the package is available separately and will undoubtedly find use as a textbook; but in an attempt to give readers the same opportunity as the authors to interpret the database and to confront the scientific problems, the boxed set also contains 14 maps at a uniform scale of 1:2.5 million, a description of the data compiled in these maps and a CD-ROM containing some of the digital information incorporated into the maps. One can always ask for more — for more of the original seismic data, or for digital versions of the maps suitable for a geographical information system — but the paper and digital databases already turn the textbook into a reference volume essential to any European geoscientist interested in the big picture.

For all that has been achieved, there is one telling omission. Despite the emphasis on imaging three-dimensional structure, not a single cross-section in *A Continent Revealed* summarizes our new knowledge of the properties of the Earth as they vary with depth beneath the EGT. Despite seven years of data acquisition, such a cross-section would certainly have large gaps or uncertainties, and would show how far the geophysical community remains from the ideal of mapping the lower crust and mantle lithosphere in the same detail as we can map the Earth's surface. But how important, before we look forward from this giant's shoulders, to know where are its feet of clay; and how else to advance further than by acknowledging the sparseness of our data? □

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Coordinating science

Peter Lipton

Reconstructing Scientific Revolutions: Thomas S. Kuhn's Philosophy of Science.

By Paul Hoyningen-Huene. University of Chicago Press: 1993. Pp. 310. £30.50, \$43.75 (hbk); £12.75, \$18.25 (pbk).

World Changes: Thomas Kuhn and the Nature of Science. Edited by Paul Horwich. MIT Press: 1993. Pp. 356. \$45, £40.50.

The Structure of Scientific Revolutions is the most widely read book ever written by a philosopher of science: it is also the most widely misunderstood. Thomas Kuhn's flowing prose is an invitation to quick and careless reading, but even careful readers have found his account of the nature and development of the sciences at once suggestive and elusive. Part of the difficulty is that each of the two central terms of his account covers an enormous and variable amount of ground. 'Paradigm' refers to anything or everything that a group of scientists share during a period of productive consensus; 'incommensurability' refers to anything or everything that makes it difficult for the members of the group to assess competing theories when the consensus breaks down.

Kuhn has clarified his position in subsequent work, but the need remains for a careful and detailed exposition, and this is just what Paul Hoyningen-Huene provides. Originally published in German three years ago, *Reconstructing Scientific Revolutions* is based on a close reading of everything Kuhn has published up to 1990, and on a year of frequent discussions with the master himself, who offers a ringing endorsement in the foreword. Hoyningen-Huene gives a scrupulous, intelligent and revealing account of just what Kuhn was up to in *Structure*, and of how his views have developed since then. *Reconstructing Scientific Revolutions* will become the Baedeker to Kuhn's philosophy of science.

What then is Kuhn's view? As a historian of science, Kuhn was struck by the degree of coordination of research strategies within particular scientific specialties during most periods of their history. The members of such a specialty share much more than theory; they agree about the choice of new problems, the way to attack them and the appropriate standards for assessing putative solutions. During such a period of 'normal science', it is as if the members of the group shared an explicit and detailed set of rules for research. Because such explicit rules do not really exist, Kuhn's problem was to find what stands in their stead.

What he found are exemplars, the central component of his capacious notion of a paradigm. Exemplars are concrete and strikingly successful problem-solutions in the group's specialty, the sort of standard examples that eventually appear in textbooks as illustrations and as chapter-end problems. They work by analogy, leading investigators to select new problems that look similar to the exemplary problems, to try techniques similar to those that worked in the exemplars, and to judge success by the standards implicit in the exemplars. In short, although they are specific in content, exemplars are general in their methodological import, focusing and coordinating the course of research. Normal science, however, never lasts. Exemplars eventually reach their limit, pointing towards new problems but no longer supporting new solutions. The result is a scientific revolution, where new exemplars replace the old ones and a different normal science tradition begins.

Kuhn's work has inspired an enormous critical literature, to which *World Changes* is the latest contribution, based on a conference held at the Massachusetts Institute of Technology in 1990. The contributors include distinguished philosophers and historians of science, as well as Kuhn himself, who gives a characteristically stimulating set of replies. The collection covers diverse aspects of Kuhn's work and several of the articles are essential reading for professional philosophers of science, but the collection as a whole does not reach the level of interest of the less polite but more provocative Kuhn-Popper confrontation, published 23 years ago under the very Popperian title *Criticism and the Growth of Knowledge* (edited by I. Lakatos and A. Musgrave, Cambridge University Press, 1970).

As the articles in *World Changes* illustrate, Kuhn's critics often press two sorts of objection. Historians and sociologists of science have argued that Kuhn underestimates the influence of external social and political factors on scientific research. Nor is this an accidental feature of his account, since the exemplar mechanism depends on the cognitive insulation of science from the rest of society. If scientists cannot choose their own problems, they cannot exploit the analogy to the exemplars that is at the heart of the Kuhnian mechanism. Most philosophers of science have found Kuhn's internalism less troublesome, but many of them have objected to his claim that the switch of exemplars that marks a scientific revolution produces a discontinuity so severe that it makes no sense to speak of scientific progress as progress towards the truth about the world.

Hoyningen-Huene gives a particularly helpful account of how Kuhn uses the innocent-looking exemplar mechanism to undermine the idea that science provides increasingly accurate and comprehensive

descriptions of a mind-independent reality. Like the great eighteenth-century philosopher Immanuel Kant, Kuhn posits a distinction between the world as it is in itself and the world as structured by our concepts. The world in itself contains the course of experience and the results of experiments, but we can know nothing about its nature. Only the other sort of world is knowable, a world we have in part created.

For Kuhn, it is the exemplars that do this creative work, because they not only guide scientists' research but also give meaning to their terms and so impose a taxonomy on nature. Kuhn, however, is Kant on wheels. For Kant, the imposed taxonomy could take only one form; but for Kuhn there is a radical change of taxonomy with each change of exemplars. (This change of meaning is the root cause of incommensurability.) Scientists are therefore not only forever ignorant of the world as it is in itself, but the world they do investigate at a particular time has only a limited lifespan, and is bound to be replaced with a new and incompatible world. Because the scientists' world changes, the notion of a single truth has no application.

Kuhn's arguments in support of changing worlds and against truth are intelligent, original and probing, but they do not in my view come close to establishing such implausible claims. They have been firmly resisted by those philosophers who hold that science does provide a fallible, but increasingly accurate, description of the world as it is in itself. Whatever one's view on the truth question, however, Kuhn's arguments cannot and will not be ignored, and these two valuable books show that it is at least possible to discover the truth about Kuhn's philosophy of science. □

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New in paperback

Wonderwoman and Superman: The Ethics of Human Biotechnology by John Harris. OUP, £8.99. Reviewed in *Nature* **356**, 203 (1992).

Chandra: A Biography of S. Chandrasekhar by Kameshwar C. Wali. University of Chicago Press, \$16.95, £13.50.

The Rise and Fall of Maya Civilisation by J. Eric S. Thompson. A classic introduction to the ancient Maya, first published in 1954. Pimlico, £11.00.

The Astronomers: A Lavishly Illustrated Journey Through Space and Science by Donald Goldsmith. St Martin's Press, \$14.95. See *Nature* **351**, 451 (1991).

Power from Steam: A History of the Stationary Steam Engine by Richard L. Hillis. CUP, £17.95, \$29.95.

Association of folding intermediates of glycoproteins with calnexin during protein maturation

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Calnexin, an endoplasmic reticulum transmembrane protein, represents a new type of molecular chaperone that selectively associates in a transient fashion with newly synthesized monomeric glycoproteins in HepG2 cells. Calnexin only recognizes glycoproteins when they are incompletely folded. Dissociation of glycoproteins from calnexin occurs at different rates and is related to the time taken for their folding, which may then initiate their differential transport rates from the endoplasmic reticulum.

It has been proposed that there is a quality control apparatus in the endoplasmic reticulum (ER) made up of molecular chaperones and folding enzymes that ensure the functional integrity of secretory proteins and regulates their transport¹⁻³. In human hepatoma HepG2 cells, large differences in the rates of transport from the ER to the Golgi apparatus have been observed for secretory proteins and the time spent in the ER represents the rate-limiting step in protein secretion^{4,5}. The search for an association of secretory proteins in HepG2 cells with the ER chaperone BiP has been unsuccessful, as have the searches for a transport receptor and for specific retention signals in secretory proteins⁵. So far such a retention mechanism has only been established for the KDEL motif and its receptor⁶.

We have previously reported the cloning of the complementary DNA of a novel calcium-binding membrane phosphoprotein of the ER, calnexin⁷. Calnexin associates with the ER membrane phosphoglycoprotein known as the signal sequence receptor SSR α (ref. 7) and has also been identified as the protein in the ER that is associated with major histocompatibility complex (MHC) class I heavy chain (where it was known as p88 (ref. 8)), and with the T-cell receptor and membrane immunoglobulin before the assembly of these multimeric complexes⁹⁻¹¹. These associations led to the proposal that calnexin might represent a molecular chaperone in the ER membrane which is selective for incompletely folded oligomeric membrane proteins newly translocated into the ER. We have examined the role of calnexin as a molecular chaperone and investigated whether it is involved in monomeric secretory protein folding in the ER. Such a function requires that calnexin bind transiently with secretory proteins and that it bind selectively with the incompletely folded form of those proteins. To test this we have used HepG2 cells, which secrete a variety of well characterized proteins^{4,12}.

We show that calnexin transiently and selectively associates with newly synthesized secretory glycoproteins but not with the non-glycosylated protein, albumin. Calnexin associates transiently with incompletely folded glycoproteins or forms a more stable association with misfolded glycoproteins. Furthermore, calnexin associates with different glycoproteins for different times and identifies the order in which these glycoproteins normally leave the ER.

Association with secretory glycoproteins

In HepG2 cells labelled with Trans³⁵S-label for 30 min followed by cell lysis and incubation with antibodies to α 1-antitrypsin, both the 52K ER form and the 55K Golgi form of α 1-antitrypsin were precipitated, with only the former being sensitive to endo H (Fig. 1a, lanes 1, 2). Quantification revealed that ~50% of the α 1-antitrypsin had reached terminal glycosylating compartments of the Golgi apparatus during this labelling period. Immunoprecipitation of cell lysates under denaturing conditions with affinity-purified calnexin antibodies (Fig. 1a, lane 3) precipitated only calnexin. But when immunoprecipitations were done with calnexin antibody under non-denaturing conditions, several proteins were coprecipitated (Fig. 1a, lane 4). The major coprecipitated proteins migrated with mobilities of 52, 66, 74, 175 and ~230K (calnexin migrates at 90K), corresponding to the mobilities of the ER forms of the major secretory glycoproteins of HepG2 cells (α 1-antitrypsin and α 1-antichymotrypsin, α -fetoprotein, transferrin, complement 3 (C3) and apoB-100, respectively). To identify the calnexin-associated proteins, we designed a sequential immunoprecipitation protocol as described in the legend to Fig. 1.

After immunoprecipitation with anti-calnexin, the associated proteins (Fig. 1a, lane 4) were eluted and immunoprecipitated with antibodies specific to the respective secretory proteins (Fig. 1a, lanes 5-11). Remarkably, α 1-antitrypsin, α 1-antichymotrypsin, transferrin, C3, apoB-100 and α -fetoprotein coimmunoprecipitated with calnexin. Albumin was not immunoprecipitated from the calnexin-eluted proteins (Fig. 1a, lane 11) although anti-albumin antibodies precipitated the protein from total cell lysates (lane 12). After 10 min of radiolabelling, 25% of newly synthesized α 1-antitrypsin, 30% of transferrin and 30% of C3 were coprecipitated with calnexin (not shown). As the efficiency of the immunoprecipitation of total cellular calnexin under these conditions was only 60%, we conclude that at least 50% of each of the newly synthesized secretory glycoproteins were calnexin associated. But radiolabelled calnexin was not detected in immunoprecipitates with antibodies to the secretory glycoproteins (Fig. 1b, lanes 1, 3) because calnexin has a relatively long half-life ($t_{1/2}$ > 24 h, not shown). Thus, these newly synthesized secretory glycoproteins enter the ER and bind with high efficiency to pre-existing unlabelled calnexin.

The non-glycosylated major secretory protein of HepG2 cells,

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albumin, was not associated with calnexin, yet the related¹³ glycosylated protein α -fetoprotein was, suggesting that only glycoproteins may bind to calnexin. We used the glycosylation inhibitor tunicamycin to evaluate calnexin association in the absence of N-linked glycosylation. Tunicamycin addition to cells led to the inhibition of glycosylation of α 1-antitrypsin and transferrin (compare Fig. 1b, lanes 1, 3 with lanes 2, 4) and these as well as most other proteins were not coimmunoprecipitated with calnexin (Fig. 1b, lanes 5, 6). That glycoproteins associated with calnexin was also demonstrated by the adsorption of calnexin-eluted proteins to concanavalin-A Sepharose. The major polypeptides associated with calnexin were those which bound to concanavalin-A Sepharose, whereas calnexin, which is not a glycoprotein⁷, was not bound (data not shown).

To evaluate whether newly synthesized glycoproteins were binding to a large network of ER-resident proteins, the sedimentation properties of calnexin-associated glycoproteins were assessed by sucrose density-gradient centrifugation. In control cells, most calnexin (Fig. 2g) was found in fractions 3 and 4 with a sedimentation coefficient of ~ 12 S. These fractions also contained the highest levels of radiolabelled proteins associated with calnexin (Fig. 2a, lanes 3, 4). The highest levels of calnexin-associated α 1-antitrypsin (52K, Fig. 2a, c) were found in fractions 2 and 3 whereas transferrin (74K, Fig. 2a) was predominantly in fractions 3 and 4; C3 (175K) was in fractions 4 and 5 and apoB-100 (~ 230 K) in fractions 5 and 6. (There were exceptions; for example, glycoproteins of 28, 30, 35K, which we have not identified, were found in fractions 3–5 of Fig. 2a.) Most calnexin-associated proteins, including apoB-100, sedimented at less than 20S (fraction 6). After tunicamycin treatment most calnexin associations were abolished (Fig. 2b). The sedimentation of the 52K band which coimmunoprecipitated with calnexin

(Fig. 2a) corresponded to that of α 1-antitrypsin (Fig. 2c) which itself sedimented further in sucrose gradients of lysates from tunicamycin-treated cells despite a lower mass of the unglycosylated protein (Fig. 2d). By contrast, newly synthesized albumin (unassociated with calnexin) showed similar sedimentation properties whether from control (Fig. 2e) or tunicamycin-treated cells (Fig. 2f). Hence, no large network of ER proteins is responsible for the calnexin associations although we cannot exclude the possibility that newly synthesized glycoproteins bind to small complexes of calnexin and resident ER proteins. Although SSR α forms a stable complex with calnexin in dog pancreatic ER⁷ we have been unable to detect this protein in HepG2 cells with available antibodies (data not shown).

Kinetics

Pulse-chase studies (Fig. 3a) demonstrated the transient association of newly synthesized proteins with calnexin. But some proteins dissociated from calnexin more quickly than others. By sequential immunoprecipitation (see legend to Fig. 1), the $t_{1/2}$ of α -antitrypsin association with calnexin was determined as 5 min (Fig. 3b, lower panel). Transferrin was associated with calnexin with a $t_{1/2}$ of ~ 35 min (Fig. 3c), whereas C3 showed an association with calnexin with a $t_{1/2}$ of ~ 25 min (Fig. 3c), as did apoB-100 ($t_{1/2} \sim 25$ min, not shown). Maximal binding to calnexin did not appear immediately after the pulse but only after 2–10 min of chase and was probably due to time needed to complete the translation of nascent polypeptide chains of larger proteins¹⁴.

We used the acquisition of endo H resistance as a measure of the time taken by secretory proteins for ER to Golgi transport. α 1-antitrypsin entered Golgi terminal glycosylating compartments as early as 10 min with a $t_{1/2}$ of ~ 20 min observed (Fig.

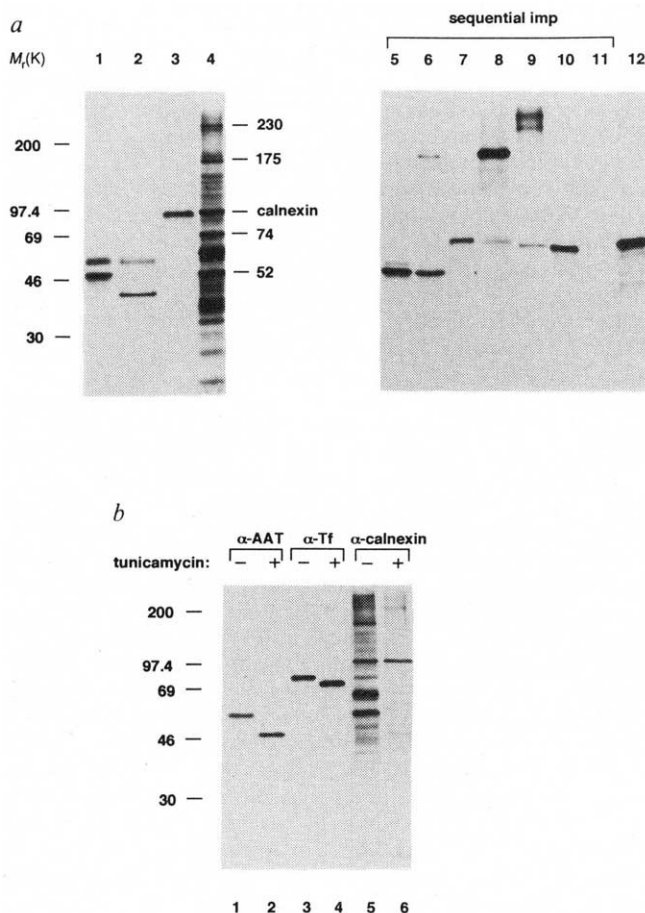


FIG. 1 Association of newly synthesized proteins with calnexin in HepG2 cells. a, HepG2 cells were radiolabelled for 30 min followed by lysis and immunoprecipitation with anti- α 1-antitrypsin (lanes 1 and 2) and either untreated (lane 1) or treated (lane 2) with endo H. Cell lysates were immunoprecipitated with anti-calnexin under denaturing (lane 3) or non-denaturing conditions (lane 4). After immunoprecipitation with anti-calnexin under non-denaturing conditions, coprecipitated proteins were eluted from protein A-agarose beads with SDS. Sequential immunoprecipitations were done with anti- α 1-antitrypsin (lane 5); anti- α 1-antichymotrypsin (lane 6); anti-transferrin (lane 7); anti-C3 (lane 8); anti-apoB-100 (lane 9); anti- α -fetoprotein (lane 10) and anti-albumin (lane 11). Lane 12 is an immunoprecipitate of cell lysates with anti-albumin. b, HepG2 cells treated with $10 \mu\text{g ml}^{-1}$ tunicamycin for 3 h (lanes 2, 4 and 6) or without (lanes 1, 3, 5) were radiolabelled for 10 min. The cell lysates were immunoprecipitated with anti- α 1-antitrypsin (lanes 1 and 2); anti-transferrin (lanes 3 and 4); and anti-calnexin (lanes 5, 6) under non-denaturing conditions followed by SDS-PAGE and fluorography. Molecular mass markers (DuPont NEN) are indicated to the left of the gels.

METHODS. Rabbit antibodies were raised to a synthetic peptide corresponding to the C terminus of calnexin (residues 555–573) and affinity purified. HepG2 cells were preincubated with methionine-free DMEM for 30 min, and then labelled with $50 \mu\text{Ci ml}^{-1}$ Tran³⁵S-label (ICN). For non-denaturing immunoprecipitations, cells were treated with 0.5 M iodoacetamide and lysed in 50 mM HEPES, pH 7.5, 200 mM NaCl (HBS) buffer containing 2% sodium cholate, 1 mM PMSF, $5 \mu\text{g ml}^{-1}$ each of aprotinin and leupeptin. Cell lysates were precleared and immunoprecipitated with affinity-purified anti-calnexin (2 h, 4°C). For immunoprecipitations under denaturing conditions, cells were lysed in HBS containing 1% SDS, and heated in boiling water for 5 min. After centrifugation, the supernatants were diluted with 10 vols HBS containing 1% Triton X-100, and immunoprecipitated with anti-calnexin as described above. Sequential immunoprecipitations were done first under non-denaturing conditions as described above. HBS (0.2 ml) containing 1% SDS was then added to the protein A-agarose beads and heated at 90°C for 3 min followed by the addition of 2 ml HBS containing 1% Triton X-100. After centrifugation, the supernatant was used for a second immunoprecipitation with specific antibodies to proteins secreted by HepG2 cells (Calbiochem) as indicated above. All immunoprecipitates were analysed on SDS-PAGE gels followed by fluorography.

3b, upper panel). For C3, a $t_{1/2}$ of 60 min was found (not shown) and for transferrin (not shown) entry was as early as 30 min, but the $t_{1/2}$ of acquisition of endo H resistance was extraordinarily long, >120 min. Therefore there was a differential lag period between the dissociation of these glycoproteins from calnexin and the acquisition of endo H resistance.

Association of glycoproteins with calnexin

The different times of association of glycoproteins with calnexin may be related to their different rates of folding in the ER. We therefore tested if only incompletely folded proteins are calnexin associated. Two experimental approaches were followed. In the first, the incorporation of the proline analogue, azetidine-2-carboxylic acid (Azc), into proteins was used to interfere with their folding. This has been used previously to demonstrate stable association of proteins with the cytosolic chaperone HSP72 (ref. 15). In HepG2 cells pulse-labelled in the presence of Azc and chased for various times in the absence of the analogue, newly synthesized proteins remained bound to calnexin (Fig. 4). Albumin in Azc-treated cells still did not associate with calnexin.

Our second approach directly examined whether calnexin associates only with incompletely folded glycoproteins during normal protein maturation. We followed the protocol in ref. 16 which defined conditions to distinguish incompletely folded intermediates during transferrin maturation in the ER of HepG2 cells. After pulse-labelling and chase, transferrin immunoprecipitates revealed in reducing gels a sharp band of 74K (Fig. 5a, upper) which was endo-H sensitive (not shown). On non-reducing gels (Fig. 5a, lower), the major portion of transferrin

migrated as a broad, diffuse set of bands at early times of chase (2–20 min). This represents the incompletely folded forms of transferrin¹⁶. Gradually, these broad bands were chased to a faster migrating sharper band corresponding to the ER folded form of transferrin with a uniform species of disulphide bonds¹⁶. About 50% of the pulse-labelled transferrin was folded after 30 min of chase. The form of transferrin that is in association with calnexin was determined by sequential immunoprecipitation. Transferrin associated with calnexin migrates as a single sharp band on reducing gels (Fig. 5b, upper) but in non-reducing gels (Fig. 5b, lower) only the broad band which represents incompletely folded transferrin is seen. No completely folded transferrin was found in association with calnexin even after 30 min of chase. Some aggregates of transferrin were also observed over the time course of the chase (Fig. 5a, lower), but these were not associated with calnexin (Fig. 5b, lower).

Discussion

Our studies demonstrate that calnexin is a new type of molecular chaperone with apparent specificity in HepG2 cells for incompletely folded glycoproteins. That newly synthesized glycoproteins bind individually to pre-existing calnexin was supported by the results of sucrose gradient analyses of calnexin-associated proteins (Fig. 2). The effect of tunicamycin and the calnexin association with α -fetoprotein but not with albumin illustrate the importance of glycosylation in the association of these proteins with calnexin. Our results explain earlier observations that in liver parenchymal cells secretory glycoproteins are ER membrane associated at early times of pulse-labelling but not

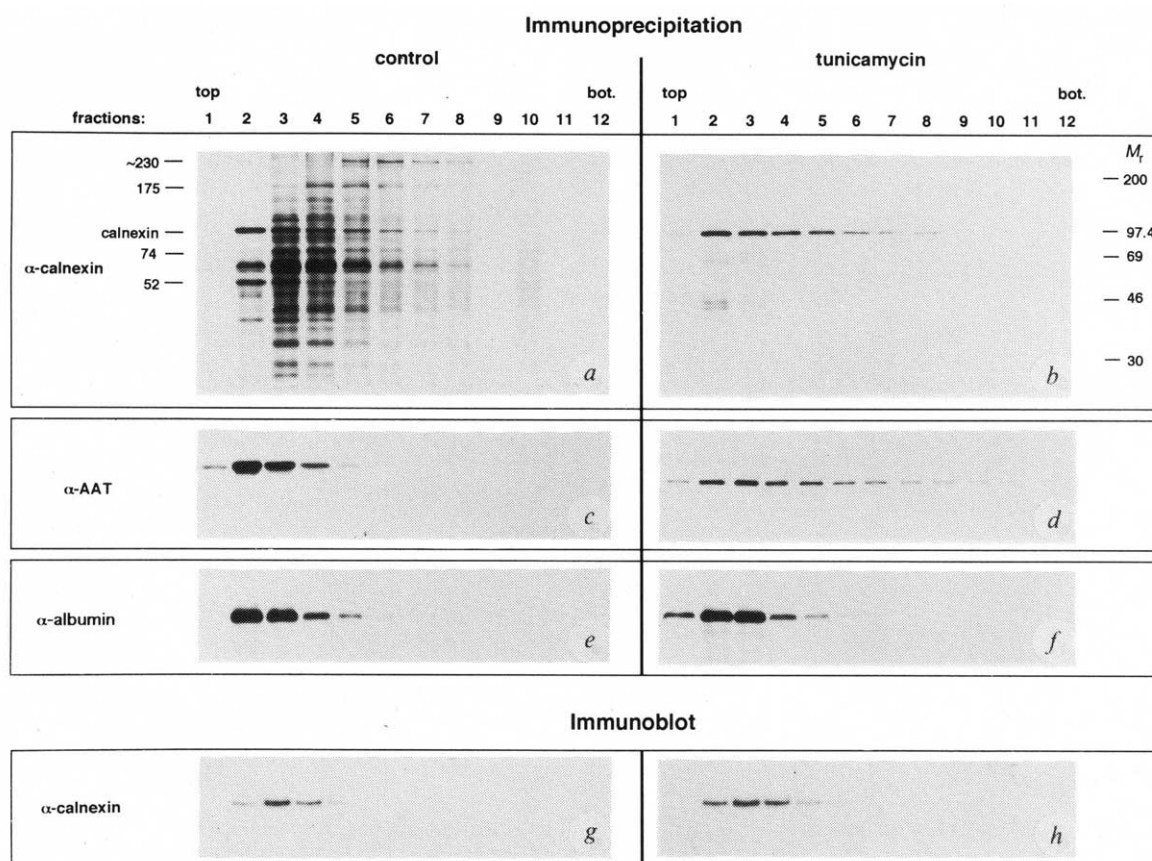


FIG. 2 Sucrose density gradient fractionation of calnexin-associated proteins. HepG2 cells without (a, c, e and g) or with tunicamycin treatment for 3 h (b, d, f and h) were radiolabelled for 10 min and then lysed in 2% cholate/HBS buffer. After centrifugation (100,000g, 20 min), supernatants were loaded onto a 5–30% (w/v) sucrose density gradient

containing 0.3% cholate in HBS buffer and centrifuged at 180,000g for 15 h at 4 °C. Fractions were immunoprecipitated under non-denaturing conditions with anti-calnexin (a and b), anti- α 1-antitrypsin (c and d) or anti-albumin (e and f). g, h, Immunoblots of the fractions probed with anti-calnexin antibody.

albumin¹⁷. The importance of *N*-glycosylation in protein folding and transport has been previously demonstrated with various proteins. Removal by mutagenesis of the glycosylation sites from model proteins results in protein misfolding^{18,19}, aggregation^{18,19}, inhibition of transport^{18,20} and association with BiP²⁰. How *N*-glycosylation is involved in calnexin association with folding intermediates is not known. Presentation of intermediates to calnexin may occur via the glycosylation apparatus of the ER. We have not demonstrated that calnexin binds directly to oligosaccharide chains or that oligosaccharides provide information on the folding state of the protein. Rather, carbohydrate side chains may render polypeptides less likely to form aggregates. The loss of carbohydrate side chains may cause conformational changes in proteins such as the surface exposure of hydrophobic regions leading to the formation of aggregates which inhibit calnexin association and favour BiP association. In the case of oligomeric transmembrane proteins⁸⁻¹¹ the potential for associations with calnexin via membrane spanning or cytosolic domains

of these proteins suggests further mechanisms for their sorting to calnexin that may be unrelated to glycosylation.

Calnexin associates with secretory proteins in a manner that can be distinguished from that reported for the ER luminal molecular chaperone BiP. Stress conditions such as heat-shock or tunicamycin treatment greatly stimulate the interaction of BiP with substrate proteins²¹⁻²⁴, but neither tunicamycin nor heat shock (result not shown) stimulates the association of proteins with calnexin. BiP-associated proteins are usually in the form of aggregates^{23,26}, but we have not observed, by sucrose gradient centrifugation, any aggregation of calnexin-associated proteins; only after tunicamycin treatment, which prevents calnexin association, was an increased sedimentation of newly synthesized α 1-antitrypsin observed, which we suggest is due to aggregation. Our studies with transferrin reveal that only incompletely folded intermediates of transferrin, apparently devoid of interchain disulphide bonds, were calnexin associated, although the interchain disulphide-bonded species existed during maturation (Fig.

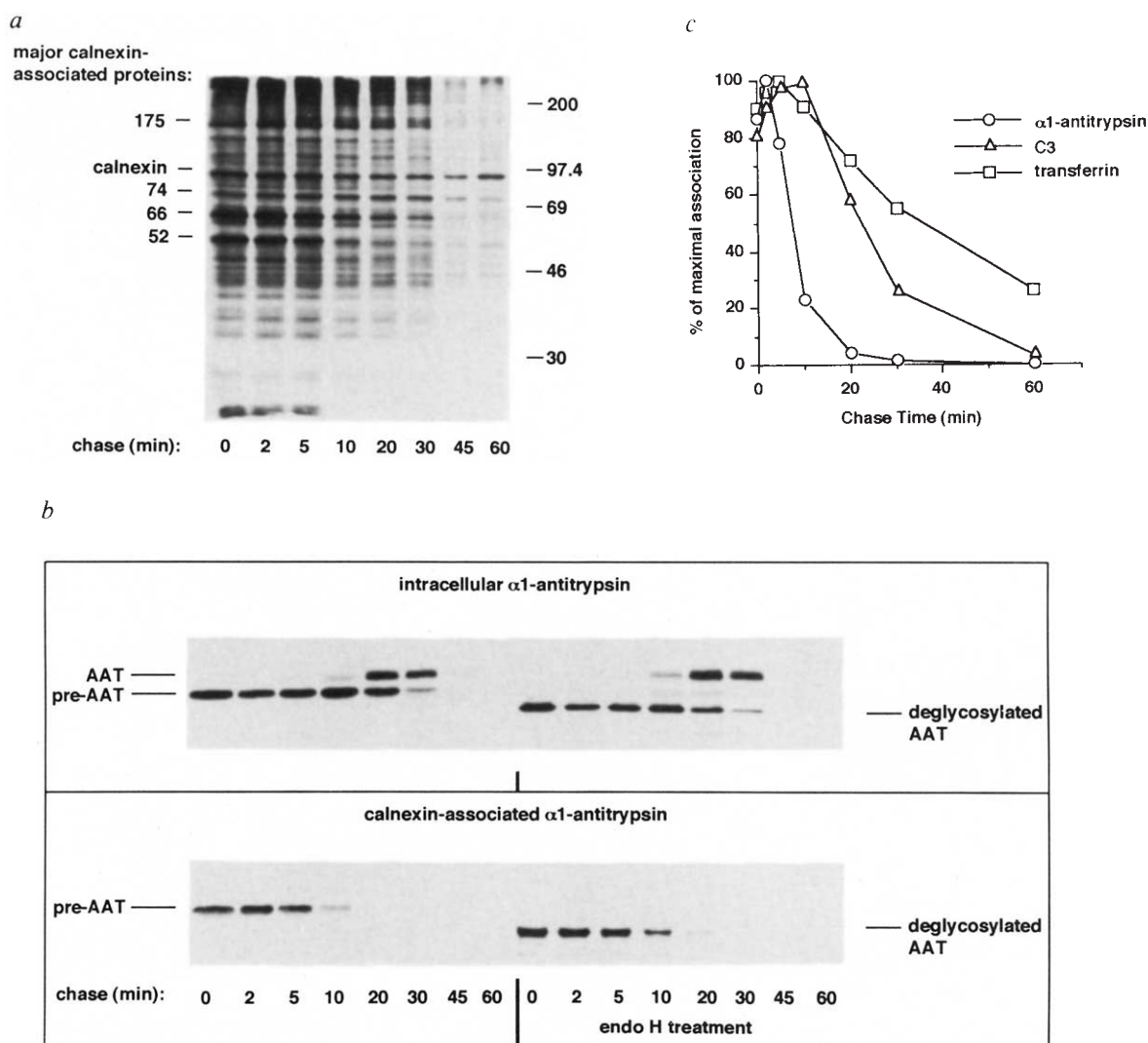


FIG. 3 Kinetics of associations of newly synthesized secretory proteins with calnexin. **a**, HepG2 were radiolabelled for 10 min, and chased in DMEM containing 1 mM methionine, 0.5 mM cysteine for the indicated times. Cell lysates were immunoprecipitated with anti-calnexin under non-denaturing conditions. **b**, After pulse-chase, cell lysates were immunoprecipitated with anti- α 1-antitrypsin (upper panel) to determine the kinetics of intracellular transport; lower panel, after cell lysates had been immunoprecipitated with anti-calnexin, calnexin-associated proteins were eluted and sequentially immunoprecipitated with anti- α 1-

antitrypsin as described in the legend to Fig. 1. The immunoprecipitates were treated with (right) or without (left) endo H at 37 °C for 1 h. **c**, After pulse-chase, cell lysates were immunoprecipitated with anti-calnexin under non-denaturing conditions. After elution of the calnexin-associated proteins, sequential immunoprecipitations were done with anti- α 1-antitrypsin, anti-transferrin or anti-C3. The immunoprecipitates were analysed by SDS-PAGE followed by fluorography. The intensity of the bands were quantified by the densitometry and expressed as a percentage of the maximum association found.

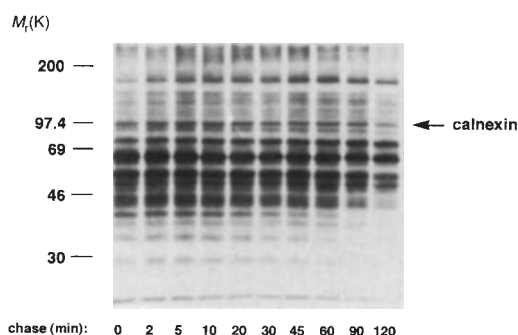


FIG. 4 Time course of the association of newly synthesized proteins with calnexin in the presence of Azc. HepG2 cells were incubated with 5 mM azetidine-2-carboxylic acid (Azc) (Sigma) in methionine-free medium for 60 min, then pulse-labelled for 10 min in the presence of 5 mM Azc and chased in the absence of the drug. At the indicated times, cells were lysed and immunoprecipitated with anti-calnexin under non-denaturing conditions as in Fig. 1. Immunoprecipitates were analysed by SDS-PAGE and fluorography. The mobility of albumin would correspond to that of the 69K marker.

5a, lower). Such interchain aggregates have been observed in other studies on protein folding *in vivo* and under defined conditions have been shown to be BiP associated²⁷. Thus we propose that calnexin recognizes different features in secretory proteins from those recognized by BiP.

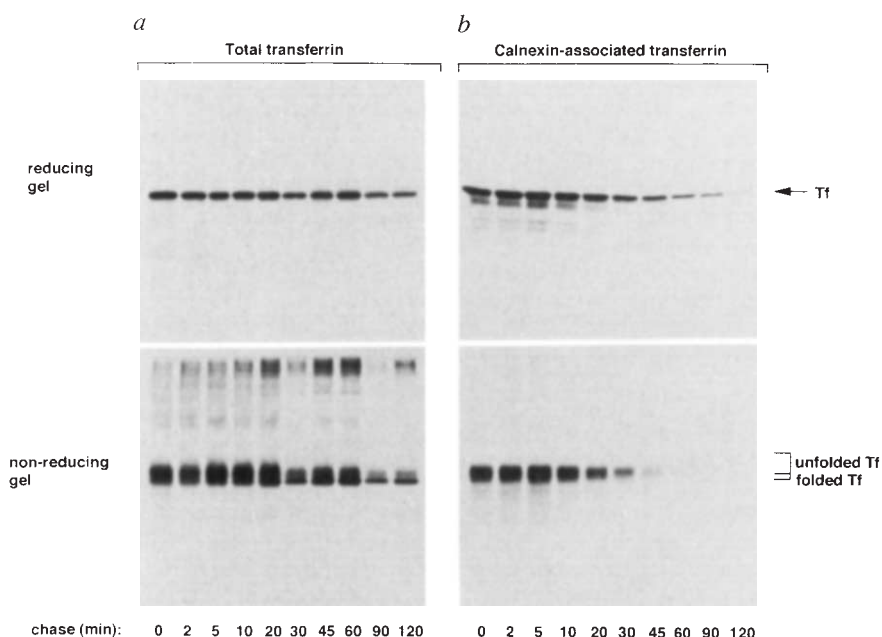
It was originally proposed that p88 (calnexin⁸) is a molecular chaperone²⁸. A differential dissociation of calnexin from different MHC class I allotypes in the same order as their differential rates of transport from the ER was observed. The differential calnexin dissociation times we observed for $\alpha 1$ -antitrypsin, C3 and transferrin are in the same order but do not account for the differential transport rates established for these proteins in ref. 4. If glycoproteins are misfolded via their incorporation of Azc, dissociation (and transport, not shown) is greatly retarded. The activities of ER enzymes involved in folding must occur during glycoprotein association with calnexin. Our studies suggest that protein disulphide isomerase activity coincides with calnexin-transferrin dissociation because transferrin dissociation is coincident with disulphide bond rearrangement.

Our results suggest that two different folding and transport pathways exist in the ER, a membrane-associated pathway and a luminal pathway. In HepG2 cells, glycosylation of nascent proteins is associated with presentation to the membrane pathway whereas non-glycosylated proteins follow the luminal pathway. Calnexin represents the major chaperone for the membrane pathway whereas other soluble chaperones are involved in the luminal pathway. Tunicamycin prevents glycoprotein presentation to the membrane-associated pathway and results in the shift of these proteins to the luminal pathway. Aggregated proteins that may be produced as side products of protein folding also go to the luminal pathway. Under normal conditions, some glycoproteins, such as $\alpha 1$ -antitrypsin, fold more rapidly on the membrane-associated pathway with tunicamycin treatment, leading to protein misfolding and inhibition of the rate of protein transport²⁹. Other glycoproteins, such as transferrin, may experience greater intrinsic difficulties in optimal folding in HepG2 cells, leading to equally slow kinetics through either the membrane or luminal pathways.

Tunicamycin treatment renders uniform the rates of transport of $\alpha 1$ -antitrypsin and transferrin, with albumin transport unaffected²⁹, a result that is predicted from the proposed role of calnexin association in initiating the differential transport rates of glycoproteins. But the tunicamycin-induced uniform transport rate corresponded to the slow kinetics of transferrin transport²⁹ because of the shift to the luminal pathway. Therefore, it is more likely that the duration of calnexin interaction with its substrates relates to the time taken for correct protein folding and that subsequent action of other downstream chaperones is required for amplification of the differential transport rates. Although a sequential chaperone action has not yet been identified in the ER, such a sequence has been demonstrated in the cytoplasm of mammalian cells³⁰, in mitochondria³¹ and in *Escherichia coli*³².

Calnexin may play a major role in the quality control mechanism for secretory glycoproteins. The retention in the ER of a number of mutant human proteins has been reviewed recently^{33,34}. These include the major form of familial hypercholesterolaemia (class 2 mutations in the LDL receptor), the major mutation causing cystic fibrosis (CFTR $\Delta F508$) as well as mutations causing juvenile pulmonary emphysema (mutations in $\alpha 1$ -antitrypsin). So far, the ER proteins responsible for this retention have not been found. A suitable candidate may be calnexin,

FIG. 5 Association of incompletely folded transferrin with calnexin. *a*, HepG2 cells were pulse-labelled for 10 min and chased for the indicated times. Cells were treated with 0.5 M iodoacetamide before lysis. Transferrin was immunoprecipitated from cell lysates with anti-transferrin, and analysed on reducing (upper panel) or non-reducing gels (lower panel) as described¹⁶. *b*, HepG2 cells were pulse-labelled and chased for the indicated times. Total cell lysates were immunoprecipitated with anti-calnexin under non-denaturing conditions. Calnexin-associated proteins were eluted from the protein A-agarose beads with SDS and sequentially immunoprecipitated with anti-transferrin antibody as described in the legend to Fig. 1. The immunoprecipitate was analysed on reducing (upper panel) or non-reducing gels (lower panel). Fluorographs in *a* and *b* were exposed to X-ray film for 4 d at -70°C . The higher order aggregates of transferrin are not calnexin associated (compare *a, b*, lower panels). They are presumed to represent interchain disulphide bonds and their significance as folding intermediates or misfolded products²⁷ is unknown.



which could retain these mutant proteins in a similar manner to that in which it retains misfolded Azc-containing glycoproteins, MHC class I heavy chain synthesized in the absence of β 2-microglobulin or T-cell receptor synthesized in the absence of

receptor α -chains^{8,11,35}. Elucidation of the role of calnexin in retaining mutant proteins responsible for these diseases is the first step in the design of pharmacological agents that will counteract their retention. □

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- Hurtley, S. M. & Helenius, A. *Rev. Cell Biol.* **5**, 277–307 (1989).
- Gething, M.-J. & Sambrook, J. *Nature* **355**, 33–45 (1992).
- Helenius, A., Marquardt, T. & Braakman, I. *Trends Cell Biol.* **2**, 227–231 (1992).
- Lodish, H. F., Kong, N., Snider, M. & Strous, G. J. A. M. *Nature* **304**, 80–83 (1983).
- Lodish, H. F. *J. biol. Chem.* **263**, 2107–2110 (1988).
- Peiham, H. R. B. A. *Rev. Cell Biol.* **5**, 1–23 (1989).
- Wada, I. *et al. J. biol. Chem.* **266**, 19599–19610 (1991).
- Ahluwalia, N., Bergeron, J. J. M., Wada, I., Degen, E. & Williams, D. B. *J. biol. Chem.* **267**, 10914–10918 (1992).
- Hochstenbach, F., David, V., Watkins, S. & Brenner, M. B. *Proc. natn. Acad. Sci. U.S.A.* **89**, 4734–4738 (1992).
- Galvin, K. *et al. Proc. natn. Acad. Sci. U.S.A.* **89**, 8452–8456 (1992).
- David, V., Hochstenbach, F., Rajagopalan, S. & Brenner, M. B. *J. biol. Chem.* **268**, 9585–9592 (1993).
- Knowles, B. B., Howe, C. D. & Aden, D. P. *Science* **209**, 497–499 (1980).
- Law, S. W. & Dugaiczky, A. *Nature* **291**, 201–205 (1981).
- Braakman, I., Hoover-Litty, H., Wagner, K. R. & Helenius, A. *J. Cell Biol.* **114**, 401–411 (1991).
- Beckmann, R. P., Mizzen, L. A. & Welch, W. J. *Science* **248**, 850–854 (1990).
- Lodish, H. F. & Kong, N. *J. biol. Chem.* **266**, 14835–14838 (1991).
- Redman, C. M. & Cherman, M. G. *J. Cell Biol.* **52**, 231–245 (1972).
- Machamer, C. E. & Rose, J. K. *J. biol. Chem.* **263**, 5955–5960 (1988).
- Matzuk, M. M. & Boime, I. *J. Cell Biol.* **106**, 1049–1059 (1988).

- Weitz, G. & Proia, R. L. *J. biol. Chem.* **267**, 10039–10044 (1992).
- Gething, M.-J., McCammon, K. & Sambrook, J. *Cell* **46**, 939–950 (1986).
- Dorner, A. J., Bole, D. G. & Kaufman, R. J. *J. Cell Biol.* **105**, 2665–2674 (1987).
- Hurtley, S. M., Bole, D. G., Hoover-Litty, H., Helenius, A. & Copeland, C. S. *J. Cell Biol.* **108**, 2117–2126 (1989).
- Machamer, C. E., Doms, R. W., Bole, D. G., Helenius, A. & Rose, J. K. *J. biol. Chem.* **265**, 6879–6883 (1990).
- Kim, P. S., Bole, D. & Arvan, P. *J. Cell Biol.* **118**, 541–549 (1992).
- Marquardt, T. & Helenius, A. *J. Cell Biol.* **117**, 505–513 (1992).
- Braakman, I., Helenius, J. & Helenius, A. *Nature* **356**, 260–262 (1992).
- Degen, E. & Williams, D. B. *J. Cell Biol.* **112**, 1099–1115 (1991).
- Lodish, H. F. & Kong, N. *J. Cell Biol.* **98**, 1720–1729 (1984).
- Brown, C. R., Martin, R. L., Hansen, W. J., Beckmann, R. P. & Welch, W. J. *J. Cell Biol.* **120**, 1101–1112 (1993).
- Manning-Kreig, U. C., Scherer, P. E. & Schatz, G. *EMBO J.* **10**, 3273–3280 (1991).
- Langer, T. *et al. Nature* **356**, 683–689 (1992).
- Amara, J. F., Cheng, S. H. & Smith, A. E. *Trends Cell Biol.* **2**, 145–149 (1992).
- Callea, F., Brisigotti, M., Fabbretti, G., Bonino, F. & Desmet, V. J. *Liver* **12**, 357–362 (1992).
- Degen, E., Cohen-Doyle, M. F. & Williams, D. B. *J. exp. Med.* **175**, 1653–1661 (1992).

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LETTERS TO NATURE

Physical surface-complexation models for sorption at the mineral–water interface

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NONE of the traditional models of surface complexation of ions at oxide–water interfaces, such as the constant-capacitance, double-diffuse-layer and triple-layer models^{1–5}, provides an explicit, quantitative treatment of ion solvation. Here I show that this process can be included quantitatively in surface-complexation theory by describing it using the Born theory of ion solvation^{6,7}. In this way, the standard Gibbs free energy of sorption can be decomposed into three terms: the standard coulombic term, a Born solvation contribution and a term intrinsic to the ion alone. Consideration of the Born solvation term shows that the equilibrium constant for sorption depends linearly on the inverse of the dielectric constant of the solid. By this means, all three contributions to the free energy can be estimated empirically or calculated theoretically. Inclusion of this physical description of ion solvation should facilitate the application of the theory of ion sorption to complex natural oxide and silicate minerals.

According to surface complexation theory, the standard Gibbs free energy of sorption of an ion j onto a solid k ($\Delta G_{r,ads,j,k}^0$) has frequently been represented^{1–4} by the sum of 'intrinsic' ($\Delta G_{int,j,k}^0$) and coulombic (ΔG_{coul}^0) terms according to

$$\Delta G_{r,ads,j,k}^0 = \Delta G_{int,j,k}^0 + \Delta G_{coul}^0 \quad (1)$$

In equation (1), the coulombic term represents the contribution to the overall free energy of adsorption owing to the interaction between the ion and the surface charge. The remaining free energy term ($\Delta G_{int,j,k}^0$) is thought to represent an 'intrinsic' contribution characteristic of both the cation and the solid. Although it has been suggested that the 'intrinsic' contribution actually consists of the sum of several effects⁵, possibly including ion solvation, the role of ion solvation has only been discussed qualitatively in the context of surface complexation theory^{1–5},

and has not been addressed quantitatively. Here I examine the term $\Delta G_{int,j,k}^0$ (or $\log K_{int,j,k}$) in more detail.

One can divide the 'intrinsic' contribution in equation (1) into a solvation contribution and a remaining term. The overall free energy of adsorption of the ion j onto the solid k can then be written as

$$\Delta G_{r,ads,j,k}^0 = \Delta G_{ii,j}^0 + \Delta G_{s,j,k}^0 + \Delta G_{coul}^0 \quad (2)$$

In this equation, the coulombic term (ΔG_{coul}^0) is preserved as defined by current usage^{1–4}, the solvation term ($\Delta G_{s,k}^0$) accounts for the role of solvation during sorption of the ion, and the remaining term ($\Delta G_{ii,j}^0$) is assumed to be a property of the ion alone. An expression for the solvation term ($\Delta G_{s,j,k}^0$) can be derived by applying Born solvation theory to the sorption process^{6,7}. Following James and Healy's treatment of Born solvation during sorption⁷, and taking account of the corresponding Helgeson–Kirkham–Flowers application of Born theory to the solvation of aqueous ions and electrolytes^{8–10}, the solvation contribution of the ion j on the solid k with dielectric constant (ϵ_k) is

$$\Delta G_{s,j,k}^0 = \Omega_j(1/\epsilon_k) + \Delta G_{ifc,j,k}^0 \quad (3)$$

where Ω_j represents a conventional Born coefficient for the ion j specifically for sorption. Following discussions in ref. 8, we can express such Born coefficients in terms of absolute Born solvation coefficients (Ω_j^{abs}) by means of the absolute solvation coefficient of the H^+ ion ($\Omega_{H^+}^{abs}$) according to

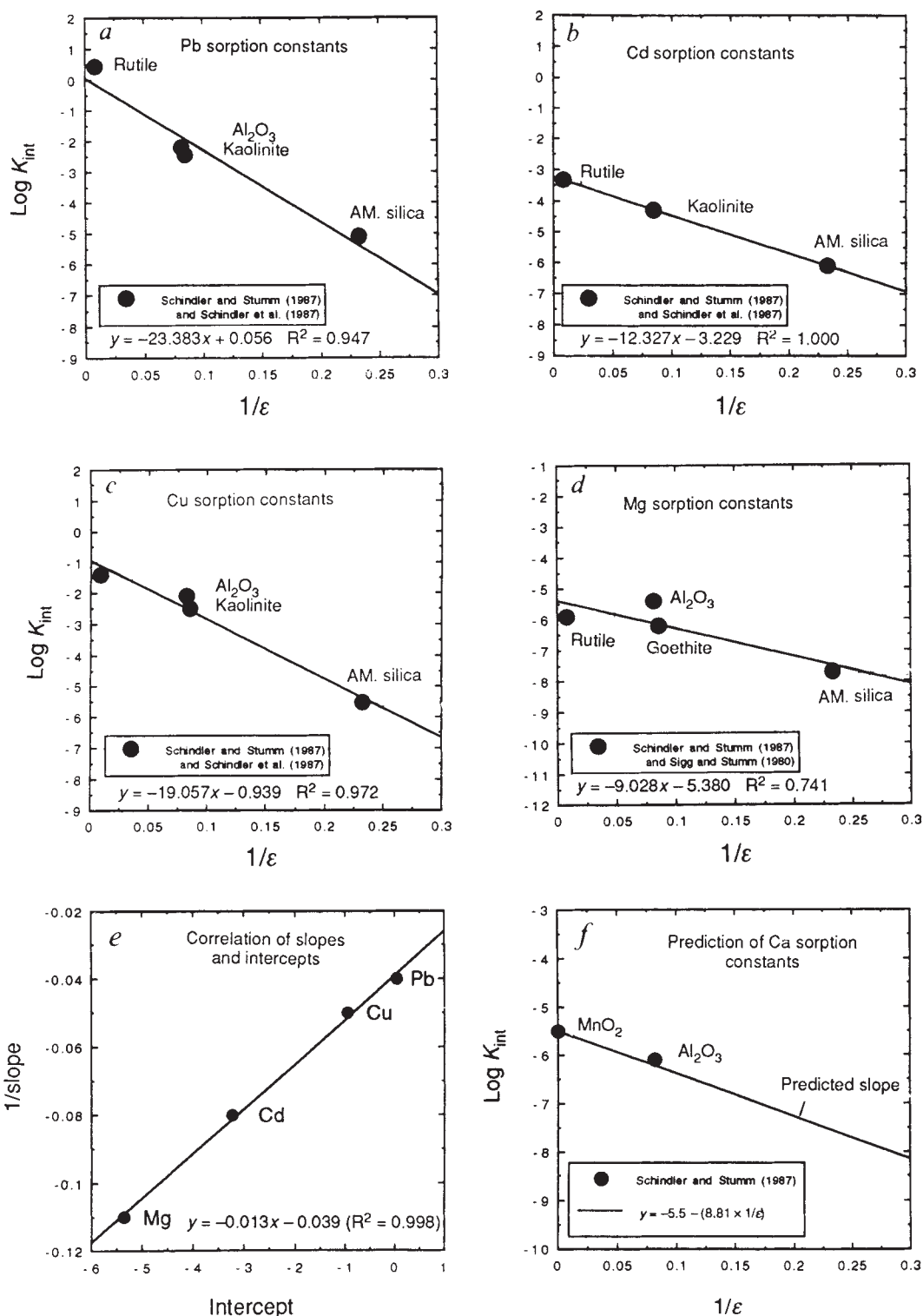
$$\Omega_j = \Omega_j^{abs} - Z_j \Omega_{H^+}^{abs} \quad (4)$$

where Z_j is the charge on the ion. The absolute solvation coefficient is in turn related to the effective electrostatic radius of the sorbed ion ($r_{c,j}$) by the equation

$$\Omega_j^{abs} = (\eta/4) Z_j^2 / r_{c,j} \quad (5)$$

where η is a constant equal to 166.027 kcal A. mol⁻¹ (see also refs 8–10). Finally, the last term in equation (3), $\Delta G_{ifc,j,k}^0$, contains contributions from the dielectric constants of the bulk solvent and the interfacial water⁷. In previous studies, the term $\Delta G_{ifc,j,k}^0$ was calculated from considerations of the surface charge on the solid⁷. However, ΔG_{coul}^0 in equations (1) and (2) also depends on surface charge^{1–4,7}. It follows that a more complete development of the theory represented by equation (2) should

FIG. 1 Correlation and prediction of 'intrinsic' equilibrium constants ($\log K_{\text{int},j,k}$) from the constant-capacitance model for sorption of an ion j on a solid k based on values of the dielectric constant of solid k (ϵ_k). a–d, linear correlation between published values of $\log K_{\text{int},j,k}$ and $1/\epsilon_k$. Values of $\log K_{\text{int},j,k}$ were taken from a summary of combined constant-capacitance and double-diffuse-layer models^{1,11}. They represent a range of experimental conditions (including highly varied ionic strengths) and a range of model assumptions concerning the nature of the solid–solution interface—for example, most do not include explicit account of sorption of supporting electrolytes^{1,11}. The following values of ϵ_k were used: rutile (120.91, the geometric mean of the principal values of rutile¹⁹), kaolinite¹⁶ (11.8), γ - Al_2O_3 (12.23, the geometric mean of the principal values of corundum¹¹), goethite and amorphous ferric hydroxide (both assumed to have the goethite value¹⁶ of 11.7), amorphous silica (4.30, the geometric mean of the principal values of quartz¹⁹). The lines represent least squares fits to the data. e, Correlation of the slopes and intercepts of the lines in Fig. 1a–d and the use of this relationship in predicting (f) the slope of the line for Ca^{2+} sorption on solids. The line shown was assumed to have an intercept determined by the experimental data for sorption of Ca^{2+} onto MnO_2 ($\epsilon = 10,000$; ref. 11) given in ref. 1. It should be noted that the line predicts a value of $\log K_{\text{int,Ca,Al}_2\text{O}_3}$ that is close to the independently derived experimental value¹.



include simultaneous evaluation of both $\Delta G_{\text{ifc},j,k}^0$ and ΔG_{coul}^0 in terms of surface charge (D.A.S., manuscript in preparation). As a first approximation, here I treat $\Delta G_{\text{ifc},j,k}^0$ separately from ΔG_{coul}^0 in equation (2), an assumption supported by the linear correlations discussed below.

Consideration of equations (1–3) and conversion to equilibrium constants suggests that published 'intrinsic' equilibrium constants can be expressed as

$$\log K_{\text{int},j,k} = -(\Omega_j/2.303RT)(1/\epsilon_k) + \log K'_{\text{ii},j} \quad (6)$$

where $\log K'_{\text{ii},j}$ corresponds to the sum of $\log K_{\text{ii},j}$ and $\log K_{\text{ifc},j,k}$. For a given ion (j) and a group of solids (k), equation (6) suggests that the dependence of the free energy of sorption on ion solvation should be manifested by a linear dependence of $\log K_{\text{int},j,k}$ on the inverse of the dielectric constant ($1/\epsilon_k$), provided that $\log K'_{\text{ii},j}$ is approximately constant.

It can be seen in Fig. 1a–d that indeed there appears to be a linear relation between published values of $\log K_{\text{int},j,k}$ and $1/\epsilon_k$. In Fig. 1a–d the fifteen experimental values of $\log K_{\text{int},j,k}$ refer mainly to the constant capacitance model^{1,11}. Although fewer

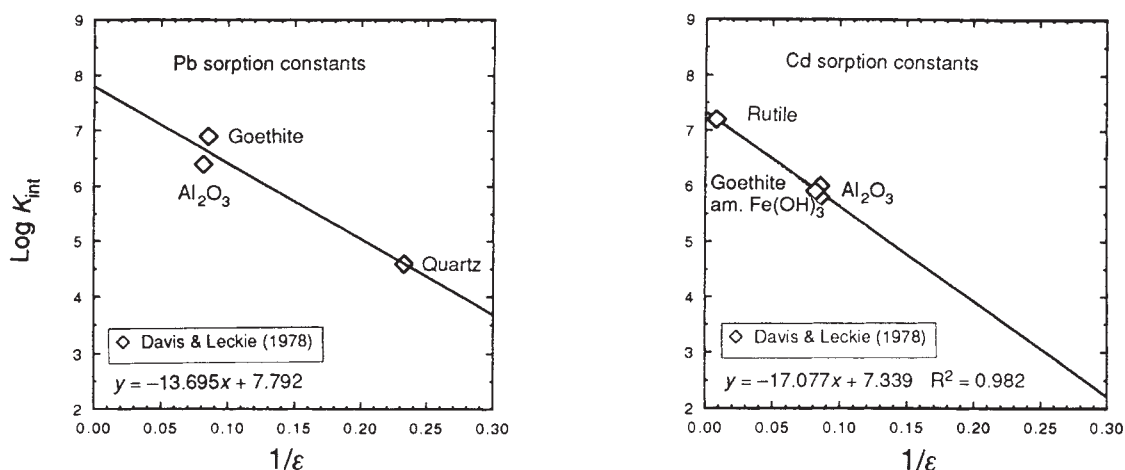
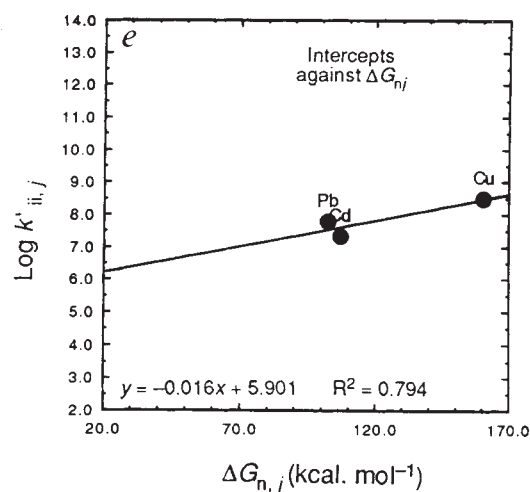
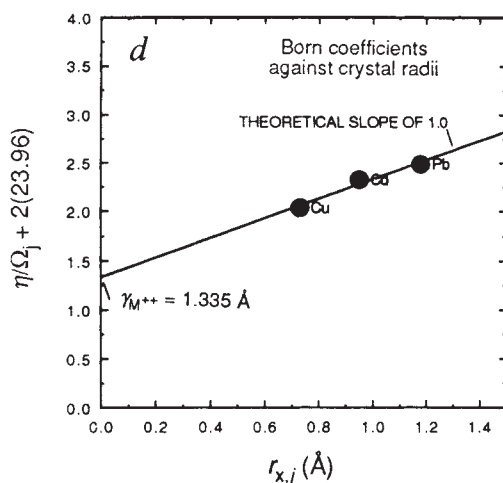
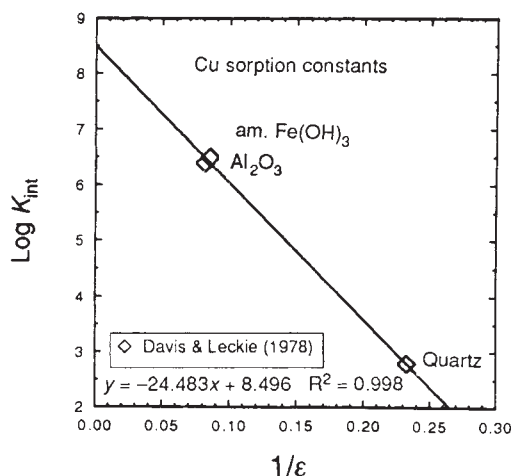


FIG. 2 Correlation and prediction of 'intrinsic' equilibrium constants ($\log K_{int,j,k}$) from the triple-layer model for sorption of an ion j on a solid k based on the values of the dielectric constant of solid k (ϵ_k). *a-c*, Linear correlation between published values of $\log K_{int,j,k}$ and $1/\epsilon_k$. Values of $\log K_{int,j,k}$ were taken from ref. 5. In contrast to the constants shown in Fig. 1*a-d*, the values derived with the triple-layer model refer to standard state conditions (with respect to the aqueous species) and include explicit account of sorption of supporting electrolytes. Values of ϵ_k used were the same as those given in the caption to Fig. 1*a-d*. The lines represent least squares fits to the data. *d*, Analysis of the slopes of the lines in Fig. 2*a-c*. Born coefficients (Ω) for Pb^{2+} , Cd^{2+} and Cu^{2+} of 18.68, 23.30 and 33.40 kcal mol $^{-1}$, respectively, derived from the slopes of the lines in Fig. 2*a-c* are correlated with crystallographic radii according to equation (8). It can be seen that a line with the theoretical slope of 1.0 is consistent with γ_{2i} equal to 1.335 Å and $\Omega_{H^+}^{abs.}$ equal to 23.96 kcal mol $^{-1}$ and an excellent fit to the three data points. *e*, Analysis of the intercepts of the lines in Fig. 2*a-c*. The intercepts from equation (6) correlate linearly with the 'non-solvation' standard free energy for ion j ($\Delta G_{n,j}^0$) (ref. 13) according to equation (9).



data are available for the triple layer model⁵, the ten data points shown in Fig. 2*a* are also consistent with a linear relationship. Taken together, Figs 1*a-d* and 2*a-c* affirm the validity of equation (6) and indicate that the $\log K_{int,j,k}$ component of $\log K'_{ii,j}$ is either constant or negligibly small. Consequently, the intercepts of the graphs depicting equation (6) in Figs 1*a-d* and 2*a-*

c are essentially independent of the solids: they are properties of the cations alone. This emphasizes that the 'intrinsic' equilibrium constant, thought to be characteristic of both the sorbing ion and the solid, should be replaced by a more fundamental ion-intrinsic constant independent of the solid, once solvation effects are explicitly taken into account (equation 6). It also appears

TABLE 1 Cation properties and predicted sorption 'intrinsic' log K values

M^{2+}	$r_{s,i}^*$	$\Delta G_{n,i}^\dagger$	$\Omega_{s,i}^\ddagger$	slope§	$\log K'_{ii,j} $	Rutile¶	Kaolin¶	Goeth.¶	Quartz¶	K-spar¶	Musc.¶	Serp.¶
Be	0.45	85.23	45.09	-33.06	7.33	7.07	4.53	4.50	-0.4	1.30	2.98	4.97
Mg	0.72	36.97	32.87	-24.10	6.49	6.30	4.45	4.43	0.89	2.09	3.32	4.77
Ca	1	-10.83	23.18	-17.00	5.73	5.59	4.29	4.28	1.77	2.63	3.49	4.51
Mn	0.82	81.26	29.12	-21.35	7.20	7.03	5.39	5.38	2.24	3.31	4.39	6.68
Fe	0.77	119.17	30.95	-22.69	7.81	7.63	5.88	5.87	2.53	3.67	4.82	6.19
Co	0.735	131.35	32.29	-23.67	8.00	7.82	6.00	5.98	2.50	3.68	4.89	6.31
Ni	0.7	136.85	33.67	-24.68	8.09	7.90	6.00	5.98	2.35	3.59	4.84	6.33
Cu	0.73	160.38	32.48	-23.81	8.47	8.28	6.45	6.43	2.93	4.12	5.33	6.77
Zn	0.745	108.23	31.9	-23.39	7.63	7.45	5.65	5.63	2.19	3.36	4.56	5.96
Sr	1.16	-24.41	18.62	-13.65	5.51	5.40	4.35	4.34	2.34	3.02	3.71	4.54
Cd	0.95	106.74	24.74	-18.14	7.61	7.47	6.07	6.06	3.39	4.30	5.22	6.31
Sn	1.11	106.28	19.98	-14.65	7.60	7.49	6.36	6.35	4.19	4.93	5.67	6.55
Ba	1.36	-36.73	13.69	-10.03	5.31	5.23	4.46	4.46	2.98	3.48	3.99	4.60
Eu	1.17	-20.50	18.36	-13.46	5.57	5.47	4.43	4.42	2.44	3.12	3.80	4.61
Hg	1.02	159.07	22.58	-16.55	8.45	8.32	7.04	7.03	4.60	5.43	6.27	7.26
Pb	1.18	102.10	18.09	-13.27	7.53	7.43	6.41	6.40	4.45	5.11	5.79	6.59
Ra	1.39	-40.06	13.01	-9.54	5.26	5.19	4.45	4.44	3.04	3.52	4.01	4.58
UO ₂	0.754	-85.15	31.56	-23.14	4.54	4.36	2.58	2.56	-0.8	0.32	1.49	2.89

All equilibrium constants in this table refer to the reaction $SO_4^{2-} + M^{2+} = (SOM)^+$, where M^{2+} is a divalent metal, and are consistent with only the triple-layer model¹⁵.

* Crystallographic radius in Ångströms¹³.

† Standard 'non-solvent' free energy in kcal mol⁻¹ (ref. 13).

‡ Conventional Born solvation coefficient for solvation calculated with equations (4), (5) and (7).

§ Slope term in equation (6) and Fig. 2.

|| Intercept term in equation (6) calculated with equation (9).

¶ Calculated values of $\log K_{int,j,k}$ (equation 6) for sorption with the triple-layer model on rutile, kaolinite, goethite, quartz, K-feldspar (microcline), muscovite and serpentine, using values of the dielectric constant equal to 120.91, 11.8, 11.7, 4.3, 5.48, 7.6 and 14.0, respectively^{16,19}.

that Figs 1a-d and 2a-c could be widely used to estimate values of $\log K_{int,j,k} \pm 0.3$ for sorption of Pb^{2+} , Cd^{2+} , Cu^{2+} and Mg^{2+} onto many other minerals for which there are at present no sorption data.

In addition, the slopes and intercepts of the $\log K_{int,j,k}$ against $1/\epsilon_k$ correlations shown in Figs 1a-d and 2a-c vary systematically. In Fig. 1e it can be seen that the inverses of the slopes correlate linearly with the intercepts from Fig. 1a-d. Taking

advantage of this, and using data for sorption of Ca^{2+} onto MnO_2 (ref. 1), resulted in the predicted slope of the line for Ca^{2+} sorption also shown in Fig. 1f. It can be seen in Fig. 1f that the predicted line passes close to the independently measured datapoint for sorption of Ca^{2+} onto Al_2O_3 . Similarly, equation (6) indicates that the slopes from Fig. 2a could be used to calculate values of Ω_j for Pb^{2+} , Cd^{2+} and Cu^{2+} , which should change systematically with $1/r$ according to Born theory^{7,8}. I now

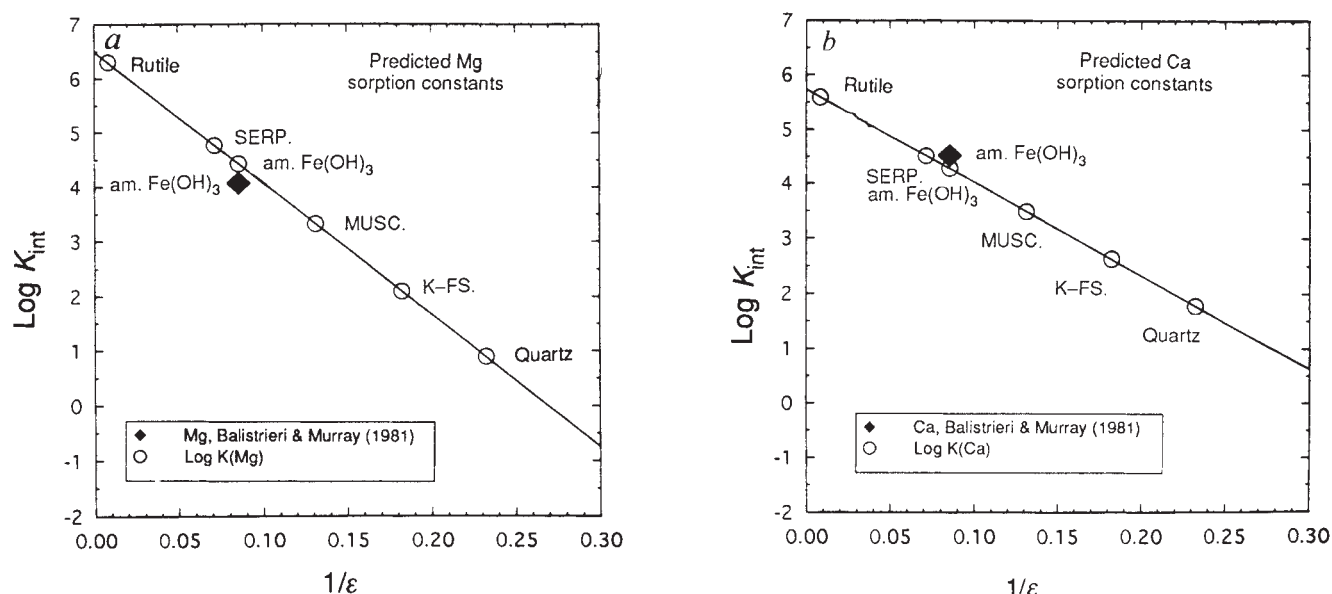


FIG. 3 Predicted values of $\log K_{int,j}$ referring to the triple-layer model for sorption of Mg^{2+} (a) and Ca^{2+} (b) onto a variety of minerals with a wide range of dielectric constants (open symbols). These predictions were made with equations (4-9) as summarized in Table 1. The solid

symbols show independently derived experimental values for amorphous ferric hydroxide¹⁵. The excellent agreement between predicted and measured values clearly shows that predictions of sorption can now be made even in the complete absence of experimental data.

assume that $r_{e,j}$ in equation (4) is related to crystallographic radius ($r_{x,j}$)¹² by a function of the charge on the cation γ_{Zj} according to⁸

$$r_{e,j} = r_{x,j} + \gamma_{Zj} \quad (7)$$

It is then possible to combine equations (5) and (7) to yield Ω_j as a function of $r_{x,j}$ with $Z_j=2$ by writing

$$\eta/(\Omega_j + 2\Omega_{H^{2+}}^{abs}) = r_{x,j} + \gamma_{2+} \quad (8)$$

According to equation (8), the function $\eta/(\Omega_j + 2\Omega_{H^{2+}}^{abs})$ plotted against $(r_{x,j} + \gamma_{2+})$ should result in a line with a slope of 1.0 and intercept of γ_{2+} for a given best fit value of $\Omega_{H^{2+}}^{abs}$. The systematic relations depicted in Fig. 2 are consistent with equations (4), (5), (7) and (8), and enable theoretical estimation of Born coefficients based solely on crystallographic radii (Table 2). The estimated values for Ω_j of Pb^{2+} , Cd^{2+} and Cu^{2+} in Table 2 differ by less than 1.4 kcal mol⁻¹ from the regression values (Fig. 2), which results in uncertainties of about ± 0.2 in $\log K_{int,j,k}$.

Because the intercepts of the lines shown in Fig. 2a–c ($\log K_{ii,j}^0$) reflect properties of the cations alone, it might be expected that they would vary systematically with the free energies of the cations (compare refs 13 and 14). It can be seen in Fig. 2e that the intercepts correlate linearly with the free energy of the j th aqueous ion corrected for a Born solvation contribution ($\Delta G_{n,j}^0$) (ref. 13) according to

$$\log K_{ii,j}^0 = (0.0160)\Delta G_{n,j}^0 + 5.901 \quad (9)$$

It now follows from equations (4), (5), (7) and (9) that both the slopes and intercepts in equation (6) can be independently predicted. As a test of this, predicted values of $\log K_{int,j}$ for Mg^{2+} , Ca^{2+} and amorphous ferric hydroxide are plotted in Fig. 3 for comparison with independent experimental values¹⁵. The excellent agreement supports the use of equations (4–9). Consequently, values of $\log K_{int,j,k}$ for all the divalent cations in Table 2 were predicted for three commonly studied solids, TiO_2 (rutile), $FeOOH$ (goethite), and SiO_2 (quartz), and for K-feldspar, biotite and muscovite, minerals for which sorption models have yet to be derived (Fig. 3). Available dielectric constants for more than 100 minerals^{16,17} permit at least two thousand such estimates for sorption of divalent metals consistent with the triple-layer model.

My results clearly demonstrate the advantages of expanding existing surface complexation models to include a physical description of ion solvation. The expanded models can thus be termed physical surface-complexation models. The triple-layer model appears to be particularly well suited to this, because the experimental results for Pb^{2+} , Cd^{2+} , Cu^{2+} , Mg^{2+} and Ca^{2+} can be closely reproduced by calculation using physical surface-complexation theory. Further developments should result in a similar treatment for ions with other charges (D.A.S., manuscript in preparation). In addition, because the dielectric constant is a tensor¹⁷, the new physical surface-complexation theory can be expected to provide insight into the differential sorption on the crystal faces of strongly anisotropic crystals, such as mica¹⁸. □

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1. Schindler, P. W. & Stumm, W. in *Aquatic Surface Chemistry: Chemical Processes at the Particle-Water Interface* (ed. Stumm, W.) 83–110 (Wiley, New York, 1987).
2. Stumm, W. & Morgan, J. J. *Aquatic Chemistry: An Introduction Emphasizing Chemical Equilibria in Natural Waters* 1–780 (Wiley, New York, 1981).
3. Davis, J. A. & Kent, D. B. in *Mineral-Water Interface Geochemistry* (eds Hochella, M. F. Jr & White, A. F.) 177–259 (Min. Soc. Am., Washington DC, 1990).
4. Dzombak, D. A. & Morel, F. M. M. *Surface Complexation Modelling* 1–393 (Wiley, New York, 1990).
5. Davis, J. A. & Leckie, J. O. *J. Colloid Inter. Sci.* **67**, 90–107 (1978).
6. Andersen, T. N. & Bockris, J. O. *Electrochim. Acta* **9**, 347–371 (1964).
7. James, R. O. & Healy, T. W. *J. Colloid Inter. Sci.* **40**, 65–81 (1972).
8. Helgeson, H. C. & Kirkham, D. H. *Am. J. Sci.* **276**, 97–240 (1976).
9. Helgeson, H. C., Kirkham, D. H. & Flowers, G. C. *Am. J. Sci.* **281**, 1241–1516 (1981).
10. Shook, E. L. & Helgeson, H. C. *Geochim. cosmochim. Acta* **52**, 2009–2036 (1988).
11. Schindler, P. W., Liechti, P. & Westall, J. C. *Neth. J. agric. Sci.* **35**, 219–230 (1987).
12. Shannon, R. D. & Prewitt, C. T. *Acta crystallogr.* **B25**, (1969).

13. Sverjensky, D. A. & Molling, P. A. M. *Nature* **356**, 231–234 (1992).
14. Sverjensky, D. A. *Nature* **358**, 310–313 (1992).
15. Balistrieri, L. S. & Murray, J. W. *Am. J. Sci.* **281**, 788–806 (1981).
16. Olhoeft, G. R. in *Physical Properties of Rocks and Minerals* (eds Touloukian, Y. S., Judd, W. R. & Roy, R. F.) 257–330 (McGraw-Hill, New York, 1981).
17. Shannon, R. D. & Rossman, G. R. *Phys. Chem. Miner.* **19**, 157–165 (1992).
18. Ilton, E. S. & Veblen, D. R. *EOS* **74**, 323 (1993).
19. Weast, R. C. *CRC Handbook of Chemistry and Physics* (CRC, Boca Raton, Florida, 1984).

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Decrease in the growth rates of atmospheric chlorofluorocarbons 11 and 12

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THE discovery of the Antarctic ozone hole¹ in 1985 led to international efforts to reduce emissions of ozone-destroying chlorofluorocarbons². These efforts culminated in the Montreal Protocol³ and its subsequent amendments, which called for the elimination of CFC production by 1996. Here we focus on CFC-11 (CCl_3F) and CFC-12 (CCl_2F_2), which are used for refrigeration, air conditioning and the production of aerosols and foams⁴, and which together make up about half of the total abundance of stratospheric organic chlorine⁵. We report a significant recent decrease in the atmospheric growth rates of these two species, based on measurements spanning the past 15 years and latitudes ranging from 83° N to 90° S. This is consistent with CFC-producers' own estimates of reduced emissions^{6,7}. If the atmospheric growth rates of these two species continue to slow in line with predicted changes in industrial emissions, global atmospheric mixing ratios will reach a maximum before the turn of the century, and then begin to decline.

We report here the recent change in growth rates of tropospheric CFC-11 and -12, based on 15 years of data from flask samples and *in situ* instruments located at the monitoring stations of the National Oceanic and Atmospheric Administration's (NOAA) Climate Monitoring and Diagnostics Laboratory (CMDL). These stations are located at Pt Barrow, Alaska, 71.3° N, 156.6° W, elevation 8 m; Mauna Loa, Hawaii, 19.5° N, 155.6° W, 3,397 m; Cape Matatula, American Samoa, 14.3° S, 170.6° W, 77 m, and South Pole, Antarctica, 90° S, 2,841 m. Cooperative sampling sites were also employed, located at; Niwot Ridge, Colorado, 40.1° N, 105.6° W, 3,472 m; Alert, Canada, 82.5° N, 62.3° W, 210 m, and Cape Grim Baseline Air Pollution Station, Australia, 40.7° S, 144.8° E, 94 m. Starting in 1977, paired samples were collected each week in electropolished, stainless steel flasks (with volumes of either 300 or 850 ml) at the four NOAA/CMDL stations and Niwot Ridge. After 1980, flask pairs at South Pole were collected once a week only during

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the austral summer when the station was open. Collection of flask pairs began at Alert in 1988, and at Cape Grim in 1991. All flasks were shipped back to Boulder for analysis by electron capture gas chromatography (EC-GC)^{8,9}. In 1987 our flask programme was complemented by automated *in situ* measurements^{9,10} (also performed by EC-GC) at the four NOAA/CMDL baseline observatories and Niwot Ridge. Measurements are reported as dry gas mole fractions or mixing ratios relative to the NOAA/CMDL 1992 gravimetric calibration scale¹⁰.

Figure 1 shows monthly means of the mixing ratios for atmospheric CFC-11 and -12 measured from more than 4,980 flask samples collected at NOAA/CMDL and cooperative stations between January 1977 and March 1993. The most obvious feature of the data is the levelling off of the mixing ratios, particularly CFC-11. Additional support for the slowdown of the atmospheric growth rates is presented in Fig. 2, which displays data collected (using *in situ* EC-GC measurements) at two sea-level sites, Pt Barrow and American Samoa. The seasonal patterns observed in the CFC data are primarily the result of transport. For Pt Barrow, the peak mixing ratios observed from November to March are the result of trapped cold air in the polar tropospheric vortex which restricts ventilation with cleaner air from the low latitudes¹¹. In contrast, the CFC maximum at American Samoa occurred during the period (November to March) of highest frequency of northwesterly flow which transports air masses enriched with CFCs from the northern hemisphere¹². It is important to note that the amplitude of the seasonal cycle for both CFCs has also decreased with time and that this decrease is consistent with reduced emissions.

The levelling off of mixing ratios after 1989 is a direct result of the reduced production of CFC-11 and -12 reported recently⁷ by the CFC producers. DuPont scientists estimated the emissions¹³ of both CFCs produced in the past, and those expected in the future, including the 1996 phaseout deadline. The procedures employed were published by Gamlen *et al.*⁴ and are based on production surveys, estimates for production in non-surveyed countries, and market analyses for use after 1990. Using these new data for worldwide emissions and an approach similar to our one-box model¹⁴, we calculated the annual mean mixing ratios of the CFCs for both hemispheres from 1930 to 2000 with a two-box, finite-increment model that describes the mean change in the tropospheric mixing ratio for a one-year interval. The differential equations for the mean annual change in the mixing ratios are for the northern hemisphere (n):

$$\frac{dX_n}{dt} = \frac{2\gamma f}{n_a} E - \frac{X_n}{\tau} - \frac{\Delta X_{ns}}{\tau_{ns}} \quad (1)$$

and for the southern hemisphere (s):

$$\frac{dX_s}{dt} = \frac{2(1-\gamma)f}{n_a} E - \frac{X_s}{\tau} + \frac{\Delta X_{ns}}{\tau_{ns}} \quad (2)$$

where X is the mean tropospheric mixing ratio in that hemisphere; γ is the ratio of the emissions in the northern hemisphere to the total emissions (~ 0.95); f is the fraction of total atmospheric CFC in the troposphere divided by the fraction of the total atmospheric mass of the troposphere¹⁴, (because f has a latitudinal dependence, means that were weighted by the cosine of the latitude were calculated as 1.10 ± 0.02 (one standard deviation, s.d.) for CFC-11 and 1.06 ± 0.02 for CFC-12 from measurements taken aboard aircraft and balloons^{15,18}, and spacecraft¹⁹); E is the emission rate (mol yr^{-1}); n_a is the total mass of the atmosphere (moles); τ is the mean atmospheric lifetime (yr); τ_{ns} is the mean interhemispheric exchange time²⁰ (1.1 yr), and ΔX_{ns} is the mean interhemispheric difference. The best fits of the observed mixing ratios in Fig. 1 were obtained with mean atmospheric lifetimes of 55 (+8, -9, 1 s.d.) yr for CFC-11 and 140 (+60, -35) yr for CFC-12. Thus using projected emissions¹³ estimated by DuPont, the model predicts max-

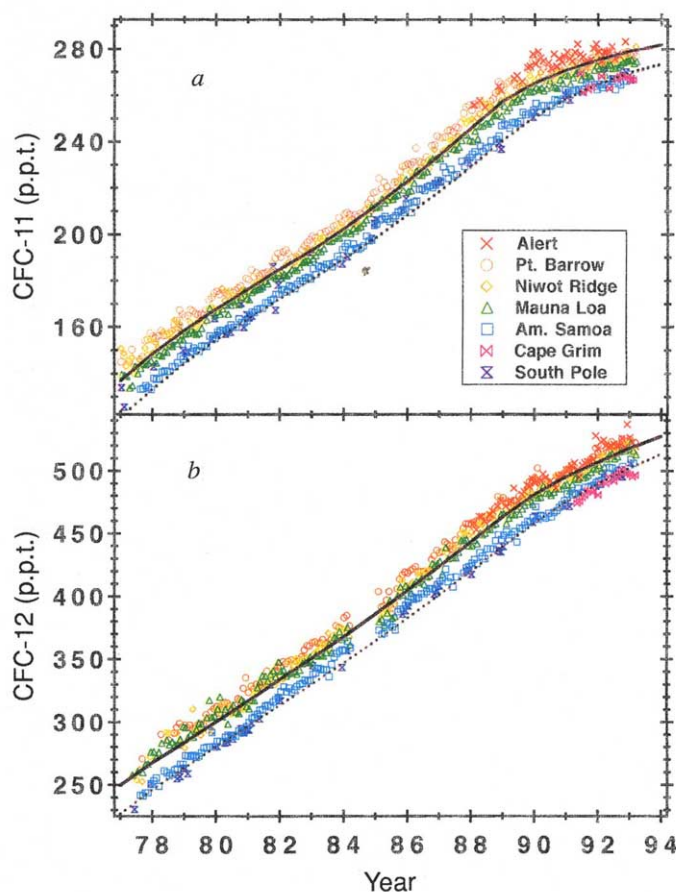


FIG. 1 Monthly means of the observed dry mixing ratio (p.p.t.) measured in flask pairs from NOAA/CMDL sites (Pt Barrow, Alaska, ($\circ$); Mauna Loa, Hawaii, (Δ); Cape Matatula, American Samoa, ($\square$); and South Pole ($\times$)) and cooperative stations (Niwot Ridge, Colorado, ($\diamond$); Alert, Canada, ($\times$); and Cape Grim, Australia, ($\times$)) for (a) CFC-11 and (b) CFC-12. Data greater than 3 standard deviations away from the fitted curves (that included a fourth-order polynomial and simple harmonic series) were eliminated in order to remove readings affected by local pollution for each station. The gap in the CFC-12 record during 1984–85 was due to a GC problem that affected only that CFC. The mean northern hemispheric (solid) and southern hemispheric (dashed) mixing ratios of each CFC in the troposphere were calculated for each year from emission estimates and our two-box model equations (1, 2). From the period of 1977 to 1985, we relied on the calibration scale of Rasmussen³⁴ which did not drift from 1978 to 1983. However, our secondary calibration tanks¹⁰ did drift at a small rate against his scale, and all mixing ratios were adjusted to correct for this drift. The uncertainty of the drift measurement during that period could only affect the observed growth rates by a small amount that is no more than ± 0.5 p.p.t. yr^{-1} . After 1985, CMDL prepared and used an independent set of gravimetrically calibrated standards, and adjusted the Rasmussen scale to our NOAA/CMDL 1992 scale¹⁰. Our absolute calibration techniques are described by Novelli *et al.*³⁵ for CO at p.p.b. levels and by Montzka *et al.*³⁶ for HCFC-22 at p.p.t. levels. From 1986 to the present, calibration tanks were routinely remade (agreed to better than 1%) and the drift of our secondary standards was statistically insignificant within our experimental precision. Our scales for CFC-11 and CFC-12 are, respectively, $3.2 \pm 1.1\%$ lower, and $3.6 \pm 1.3\%$ higher, than the Rasmussen scale³⁴ reported by Cunnold *et al.*²⁵. The differences were based on a comparison of our flask data with their *in situ* observations at a jointly monitored station at Samoa during 1978–83. Results from a recent laboratory comparison of two cylinders showed that our scale for CFC-11 is 0.1% higher, and for CFC-12 is 1.3% higher, than the 1986 scale of R. F. Weiss³⁷.

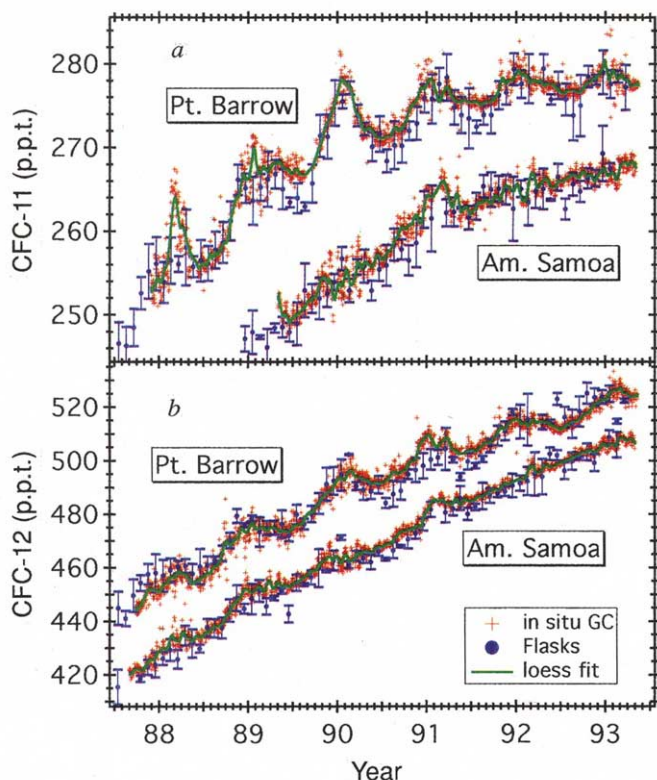


FIG. 2 Daily mean mixing ratios (p.p.t.) from *in situ* GCs (+) and monthly means from flask pairs (●) with standard deviations (error bars) for (a) CFC-11 and (b) CFC-12 from the sea-level stations of Pt Barrow (top) in the northern hemisphere and American Samoa (bottom) in the southern hemisphere. Each flask point represents the average of 4 flask pairs that were measured three times for each flask by EC-GC, with an average monthly standard deviation of ± 2.3 p.p.t. for CFC-11 and ± 3.9 p.p.t. for CFC-12. One daily mean from an *in situ* GC represents the average of about 16 individual measurements with an average standard deviation of ± 1.4 p.p.t. for CFC-11 and ± 2.7 p.p.t. for CFC-12. Data selection involved removing outliers greater than 3 s.d. away from the loess fit for each station. Seasonal transport at the sea-level stations is highlighted by the solid lines, calculated using a loess regression²¹ and a one-month filter width on the *in situ* data.

imum mixing ratios for the northern hemisphere as ~ 290 parts per trillion, p.p.t. of CFC-11 in 1998 and ~ 555 p.p.t. of CFC-12 in 1999.

For further analysis of the growth rates for the CFCs, flask data were smoothed and filtered by the locally weighted least-squares (that is, loess regression²¹) procedure from the Dataplot graphics and statistical package²². The period of the data set that was locally weighted was fixed at 24 months, sufficient to examine the interannual variations of the growth rate. We estimated the growth rates by differentiating the time series of smoothed and filtered mixing ratios. These values were then compared to the ratio generated by our two-box model (Fig. 3). The uncertainty of the growth rates was estimated by applying the Bootstrap technique²³ to the residuals of the loess technique.

One of the most significant features of the observed data shown in Fig. 3 is the dramatic slowdown in growth rates for both CFCs beginning in late 1989. Previous reports^{24–26} showed that the mean global growth rates between 1977 and 1984 were linearly increasing at a constant rate; our results give global rates of 9 ± 1 (1 s.d.) p.p.t. yr^{-1} for CFC-11 and 17 ± 3 p.p.t. yr^{-1} for CFC-12. Increased CFC usage during 1985–88 resulted in the observed growth rates climbing to average maxima for both hemispheres of 11 ± 1 p.p.t. yr^{-1} for CFC-11 and 19.5 ± 2

p.p.t. yr^{-1} for CFC-12. Global growth rates have decreased significantly since 1988, reaching levels of 2.7 ± 0.7 p.p.t. yr^{-1} for CFC-11 and 10.5 ± 0.3 p.p.t. yr^{-1} for CFC-12 by 1993. Perhaps the most significant fall-off of CFC-11 has occurred at the most northerly stations, Pt Barrow (Fig. 2) and Alert, where the rate of increase has essentially reached zero. This corresponds to the decrease in industrial production^{6,7} in the northern hemisphere where most of the material is used and therefore emitted. At the same time, growth in the southern hemisphere has decreased less rapidly because its growth rate is determined mainly by interhemispheric transport. The dramatic fall-off of CFC-11 is a result of its curtailed use particularly in the aerosol propellant and foam blowing applications in 1990. If these slowed growth rates are common for all compounds under regulation by the Montreal Protocol, the lower limit for the total cumulative organic chlorine, CCl_4 , introduced into the stratosphere would be less than 4.0 parts per billion (p.p.b.) (current value⁵ = 3.4 ± 0.2 p.p.b.).

Besides CFC production, other factors such as atmospheric

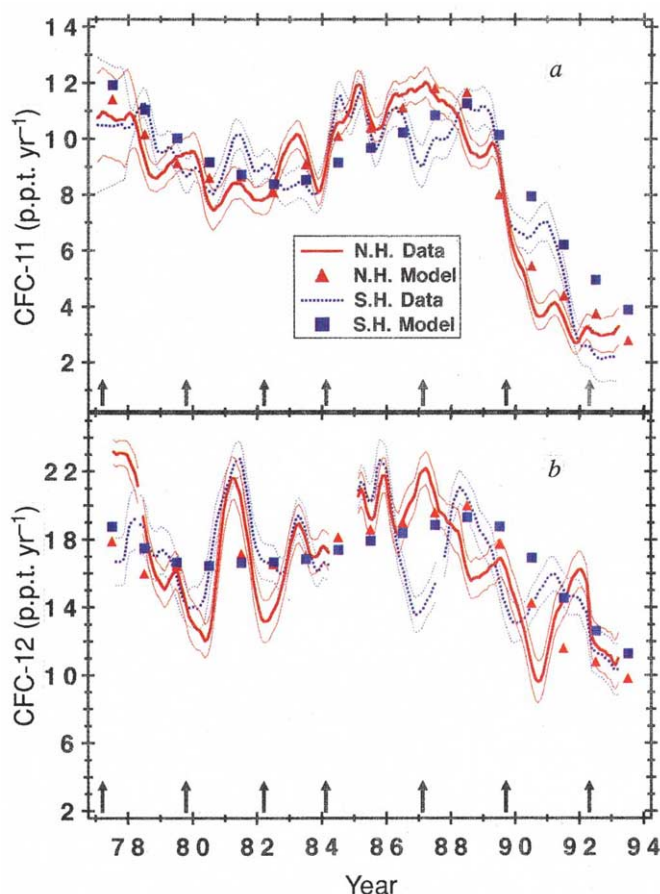


FIG. 3 Atmospheric growth rates (p.p.t. yr^{-1}) in the Northern (N.H.) and Southern hemisphere (S.H.) from flask pairs for (a) CFC-11 and (b) CFC-12. Thick solid lines are the calculated mean growth rates in the N.H. from flask values (Fig. 1) weighted by the cosine of latitude (n.b. South Pole set to latitude -67°). Thick dashed lines are the observed mean growth rates in the S.H. Growth rates were calculated using a loess regression²¹ and a 24-month filter width. Using Bootstrap procedures²³, the standard deviation of the growth rate (thin lines above and below the thick lines) of each time point was calculated by generating 50 new sets of data that randomly add or subtract the residuals to the predicted values, reapplying the loess regression to the new set, and calculating a new growth rate. Triangles represent the calculated growth rates in the N.H. by differentiating the results from the two-box model (equation (1)). Similarly, squares represent the calculated growth rates in the S.H. (equation (2)). The arrows indicate QBO events for the maximum easterly winds at 30 mb over Singapore³¹ (1°N , 104°E).

transport may affect the observed hemispheric growth rates. A statistically significant divergence of the northern and southern hemispheric growth rates occurred during the period of the El Niño-Southern Oscillation (ENSO) event of 1986–87. This ENSO had a much stronger correlation with the growth rates of both CFCs than did the event of 1982–83. ENSO events, occurring every 4–7 years²⁷, are known to affect the interhemispheric transport of trace gases and have been correlated with lower than average mixing ratios of CH₃CCl₃ at Samoa²⁸. Suppression of the westerly winds at 200 millibar (mb) in the upper troposphere which occurs during ENSOs²⁷ reduces the interhemispheric transport²⁹. The variations observed in the hemispheric growth rates for both CFCs correlate well with hemispheric variations recently noted for methane³⁰ during 1986–89. A reason that the ENSO of 1986–87 had such a strong effect on the growth rates may be the coincidences of a quasi-biennial oscillation event (QBO), typically occurring every 26 months²⁹, followed by the strongest cold event in 15 years, the La Niña²⁷ of 1988–89. Also, there is a good correlation between the maximum of easterly winds of the equatorial stratosphere³¹ (QBOs) and the drop in the southern hemispheric growth rate for both CFCs (Fig. 3). Although there is considerable evidence of tropospheric QBOs³², dynamic models also show that strong easterly winds in the stratosphere can enhance vertical transport upward from the troposphere³³ and concurrently decrease the north to south tropospheric exchange²⁹. As the mixing ratios of the CFCs have always been higher in the northern than southern hemisphere, any process that inhibits interhemispheric exchange or enhances tropospheric–stratosphere exchange will slow the growth rates in the southern hemisphere.

These decreased overall growth rates are encouraging in light of voluntary national and international efforts to limit emissions of ozone-depleting substances. Note that a recent report¹⁴ also observed decreased atmospheric growth rates for the two dominant atmospheric halons, which are used primarily in fire extinguishers, and pose a significant threat to stratospheric ozone, although less than that posed by the CFCs considered here. Furthermore, continuous monitoring of CFCs, together with accurate tracking of emissions, will help to define the chemical lifetimes of these species and may provide a more complete understanding of the transport properties of the atmosphere. □

24. Rasmussen, R. A. & Khalil, M. A. K. *Science* **232**, 1623–1624 (1986).
25. Cunnold, D. M. et al. *J. geophys. Res.* **91(D10)**, 10797–10817 (1986).
26. Elkins, J. W., Thompson, T. M., Hall, B. D., Egan, K. B. & Butler, J. H. *Antarc. J. U. S.* **23**, 76–77 (1988).
27. Trenberth, K. E. in *Teleconnections Linking Worldwide Climate Anomalies* (eds Glantz, M., Katz, R. W. & Nicholls, N.) 13–42 (Cambridge Univ. Press, 1991).
28. Prinn, R. et al. *J. geophys. Res.* **97(D2)**, 2445–2461 (1992).
29. Webster, P. J. & Holton, J. R. *J. Atmos. Sci.* **39**, 722–733 (1982).
30. Steele, L. P. et al. *Nature* **358**, 313–316 (1992).
31. *Climate Diagnostics Bulletin* June 1992, Vol. 92, No. 6 (eds Koushy, V. E., Bell, G. D. & Kopman, J. D.) 7 (U.S. Dept. of Commerce/NOAA, Washington DC, 1992).
32. Trenberth, K. E. *Mon. Weath. Rev.* **108**, 1370–1377 (1980).
33. Plumb, R. A. & Bell, R. C. *Q. J. R. Met. Soc.* **108**, 335–352 (1982).
34. Rasmussen, R. A. & Lovelock, J. E. *J. geophys. Res.* **88(C13)**, 8369–8378 (1983).
35. Novelli, P. C., Elkins, J. W. & Steele, L. P. *J. geophys. Res.* **97(D7)**, 13109–13121 (1991).
36. Montzka, S. M., Myers, R. C., Butler, J. H., Elkins, J. W. & Cummings, S. O. *Geophys. Res. Lett.* **20(8)**, 703–706 (1993).
37. Bullister, J. L. & Weiss, R. F. *Deep-Sea Res.* **35(5)**, 839–853 (1988).

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Evaluating the role of climate cooling in iceberg production and the Heinrich events

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BOND *et al.*¹ recently presented evidence for the frequent occurrence, in the Pleistocene epoch, of periods of massive iceberg discharge into the North Atlantic ocean ('Heinrich events'), each lasting a few thousand years. The cause of these events is uncertain, but one possibility¹ is repeated advances of the Laurentide ice sheet during episodes of cooler climate. Here I examine this idea, using a model of the Laurentide ice sheet driven by orbitally forced variations in insolation, with and without three additional 3,000-yr-long cooling episodes, imposed just before the occurrence of the three most recent Heinrich events (H1, H2 and H3). In the model, the cooling event preceding H1 (when the climate was relatively warm and deglaciation was about to begin) led to increased calving, but those preceding H2 and H3 (which occurred during the last ice age, when the climate was very cold) led to reduced iceberg calving. It thus seems unlikely that the Heinrich events generally reflect a direct response of the Laurentide ice sheet to climatic cooling.

The ice sheet model I use treats the Laurentide ice sheet in a simplified geometry. It calculates the evolution of the ice sheet along four flow lines that merge in Hudson Bay. Each flowline represents a sector (Fig. 1). Topography is schematically included. This means that when cooling occurs, two ice sheets start to grow on the high ground in sector 1 (Ellesmere/Baffin Land) and sector 2 (Labrador). These sheets grow towards the coast and in the direction of Hudson Bay, and eventually start to feed into sectors 3 and 4. Free mass exchange between the sectors is achieved by setting the ice thicknesses equal at the Hudson Bay centre. Although this approach implies a poorer resolution than obtained with two-dimensional models^{2,3}, I believe that it is adequate for a calculation where the main interest is in ice budgets, calving rates and runoff of melt water (here melt water is understood to be surficial melt water generated on the ice sheet—not from icebergs melting in the ocean).

Ice flow is treated in a vertically integrated formulation, as has been done in many studies of glacial cycles^{4,9}. There is no explicit calculation of ice temperature. Both sliding and internal deformation contribute to the ice flow. Sliding is allowed when melt water is produced at the surface. The grid-point spacing along each flowline is 50 km. The response of the bed simply

Received 30 June 1992; accepted 7 July 1993.

1. Farman, J. C., Gardiner, B. G. & Shanklin, J. D. *Nature* **315**, 207–210 (1985).
2. Molina, M. J. & Rowland, F. S. *Nature* **249**, 810–814 (1974).
3. *Montreal Protocol to Reduce Substances that Deplete the Ozone Layer Report, Final Report* (UN Environmental Programme, New York, 1987).
4. Gamlen, P. H., Lane, B. C., Midgley, P. M. & Steed, J. M. *Atmos. Environ.* **20(6)**, 1077–1085 (1986).
5. Prather, M. J. & Watson, R. T. *Nature* **344**, 729–734 (1990).
6. McFarland, M. & Kaye, J. A. rev. *J. Photochem. Photobiol.* **55**, 911–929 (1992).
7. *Chlorofluorocarbons (CFC's) 11 and 12 (Alternative Fluorocarbons Envir. Acceptability Study, Washington DC, 1991).*
8. Thompson, T. M., Komhyr, W. D. & Dutton, E. G. *Chlorofluorocarbon-11, -12, and Nitrous Oxide Measurements at the NOAA/GMCC Baseline Stations (16 Sept. 1973 to 31 Dec. 1979)* NOAA Tech. Rep. ERL 428-ARL 8 (NOAA Air Resources Lab., Boulder, 1985).
9. Elkins, J. W. & Rossen, R. (eds) *Summary Report 1988: Geophysical Monitoring for Climatic Change Vol. 17* (NOAA Air Resources Lab., Boulder, 1989).
10. Montzka, S. M. et al. *Summary Report 1991: Climate Monitoring and Diagnostic Laboratory Vol. 20* (eds Ferguson, E. E. & Rossen, R.) (NOAA Climate Monitoring and Diagnostic Lab., Boulder, 1992).
11. Barrie, L. A. & Hoff, R. M. *Atmos. Environ.* **18**, 2711–2722 (1984).
12. Halter, B. C., Harris, J. M. & Conway, T. J. *J. geophys. Res.* **93(D12)**, 15914–15918 (1988).
13. *Concentrations, Lifetimes, and Trends of CFCs, Halons, and Related Molecules in the Atmosphere, NASA Tech. Rep. (in the press)* (NASA, Washington DC, 1993).
14. Butler, J. H., Elkins, J. W., Hall, B. D., Cummings, S. O. & Montzka, S. A. *Nature* **359**, 403–405 (1992).
15. Goldan, P. D., Kuster, W. C., Albritton, D. L. & Schmeltekopf, A. L. *J. geophys. Res.* **85(C1)**, 413–423 (1980).
16. Fabian, P., Borchert, R., Penkett, S. A. & Prosser, N. J. D. *Nature* **294**, 733–735 (1981).
17. Fabian, P. et al. *J. geophys. Res.* **86(C6)**, 5179–5184 (1981).
18. Schmidt, U. et al. *Geophys. Res. Lett.* **18(4)**, 767–770 (1991).
19. Zander, R. et al. *J. Atmos. Chem.* **15**, 171–186 (1992).
20. Jacob, D. J., Prather, M. J., Wofsy, S. C. & McElroy, M. B. *J. geophys. Res.* **92(D2)**, 6614–6624 (1987).
21. Cleveland, W. S. *J. Amer. Stat. Assoc.* **74**, 829–836 (1979).
22. Filliben, J. J. *Comput. Graph.* **15(3)**, 199–213 (1981).
23. Efron, B. & Tibshirani, R. *Science* **253**, 390–395 (1991).

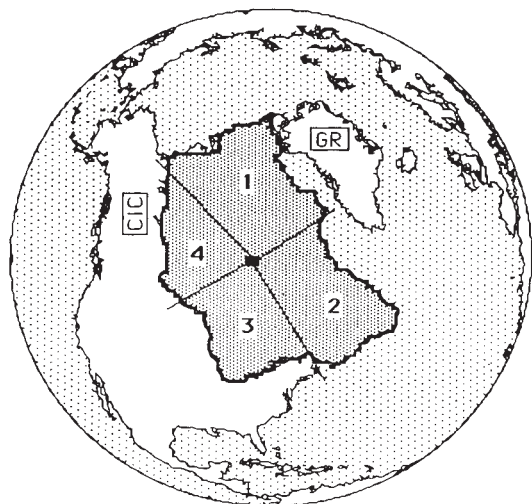


FIG. 1 Four sectors of the Laurentide ice sheet, for which mass-balance and ice-flow calculations are done separately. All sectors have runoff of melt water. Calving of icebergs occurs in sectors 1 and 2 only, when the ice edge reaches deep water. The Cordilleran Ice Complex (CIC) and the Greenland Ice Sheet (GIS), which may have interacted with the Laurentide ice sheet to some extent, are not treated in the model. Outline of ice extent is for the Late Pleistocene maximum¹⁸.

follows damped return to local isostatic equilibrium with a 5 kyr decay time.

The ice-flow model is coupled to a numerical mass-balance model^{10,11}. It calculates the mass balance from an integration of the surface energy budget through an annual cycle, given climatic input including precipitation. Dependence of the energy balance on height results from a constant atmospheric temperature lapse

rate and different radiative properties of atmosphere and cloud. The mass-balance model has been tested against observations from glaciers in widely differing climates¹². The climatic input is a function of height and distance along the flow lines, and is perturbed with series of deviations of insolation, $\delta Q(t)$, and temperature, $\delta T(t)$. Single series of these quantities are assumed to characterize the entire Laurentide ice-sheet region. $\delta T(t)$ is the departure of temperature for the summer half year. It is obtained from:

$$\delta T(t) = f_{\text{green}}(a\delta Q(t) + f_{\text{ice}}A) \quad (1)$$

Here a relates the changes of temperature to the change in insolation, including all feedbacks except those associated with atmospheric greenhouse gas concentration and ice albedo. These appear separately in equation (1). The most important feedbacks assumed to be contained in a are the water-vapour feedback and seasonal snow-cover feedback. The strength of the greenhouse feedback^{13,14} is denoted by f_{green} . The ice-albedo feedback (actually related to total land ice cover on the globe but in the present case assumed to be related only to the size of the Laurentide ice sheet) has strength f_{ice} . It is set proportional to a normalized measure of the land surface covered by glacier ice in North America (A) as calculated by the model.

I used values of f_{green} and a of 2 and $0.11 \text{ KW}^{-1} \text{ m}^2$. This implies that the varying concentration of greenhouse gases during glacial cycles doubles climate sensitivity. In the calculation of the mass-balance fields, it has been assumed that precipitation at each location varies in proportion to the saturation vapour pressure of the local air temperature. This is a first-order estimate of changes in precipitation, which finds some support from ice-core analysis¹⁵ and modelling studies^{16,17}.

Tuning was done by varying the strength of the albedo feedback (Fig. 2a). All integrations started at 125,000 years ago (zero ice volume). The best result for ice volume was obtained with $f_{\text{ice}} = 2.3$. Significantly smaller values do not produce maximum ice cover around 20,000 years ago. Larger values do not simulate the last termination (the ice sheet does not decay entirely at the beginning of the Holocene epoch). Although this model could be criticized for its simplicity in representing the Laurentide geometry, I believe that it simulates the last glacial cycle well. No strong additional destabilizing mechanisms, like extreme calving

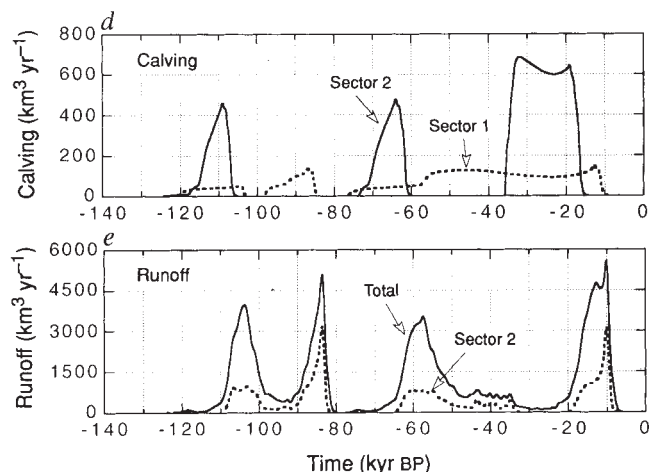
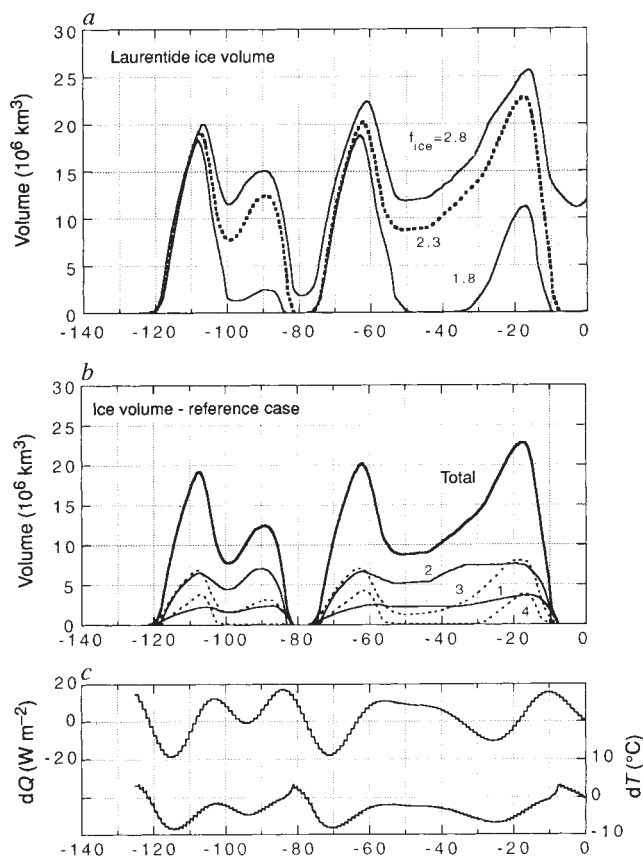


FIG. 2 a, The dependence of simulated Laurentide ice volume through the last glacial cycle on the ice feedback parameter f_{ice} . A value of 2.3 gives the best result, which is thus taken as the reference case. Ice volume in the four sectors for the reference case are shown in b. c, The forcing (summer insolation) and summer temperature, which depend on insolation and ice-sheet size. Calving rates and runoff of surficial melt water for the reference case are shown in d and e.

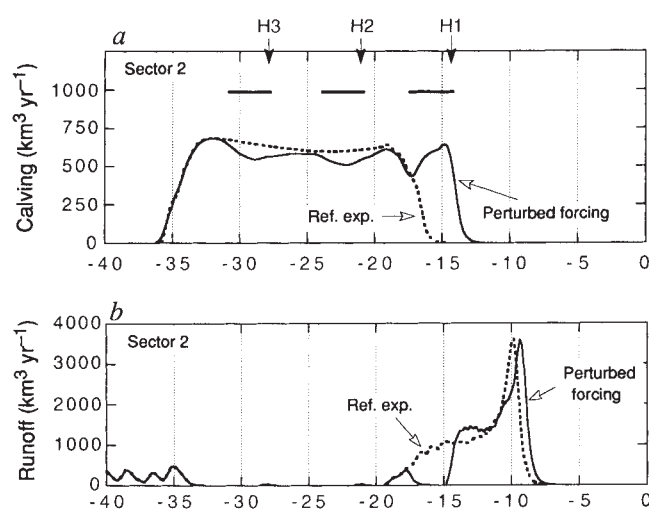


FIG. 3 Comparison of calving (a) and runoff (b) rates for the reference case and the run with imposed cooling events (labelled 'perturbed forcing'). The imposed cooling events are indicated by horizontal bars in a. Arrows mark the Heinrich layers¹. The first two cooling events lead to a reduction of calving rate, the third one to a significant increase, essentially reflecting a delay of the termination.

rates or dramatic drops in ice albedo, have to be included to make the ice sheet disappear. The rapid decay rate is the result of the realistic treatment of the mass balance, leading to melt rates much higher than would be obtained by using, for instance, the equilibrium-line concept with a fixed balance gradient. Also, the inclusion of sliding when melt water is produced helps because it makes the ice-sheet somewhat flatter and lower in the ablation zone.

A further diagnosis of the run with $f_{ice} = 2.3$, referred to as the reference case, is given in Fig. 2b–e. During full glacial conditions, sectors 2 and 3 have the largest volume (Fig. 2b), which is a consequence of the higher snow accumulation rates as compared to sectors 1 and 4. Calculated Laurentide ice volume during the last glacial maximum is about $23 \times 10^6 \text{ km}^3$. This is significantly less than values given in early reconstructions^{18,19} but in agreement with later studies²⁰ (note that the Cordilleran ice complex is not included in the present model).

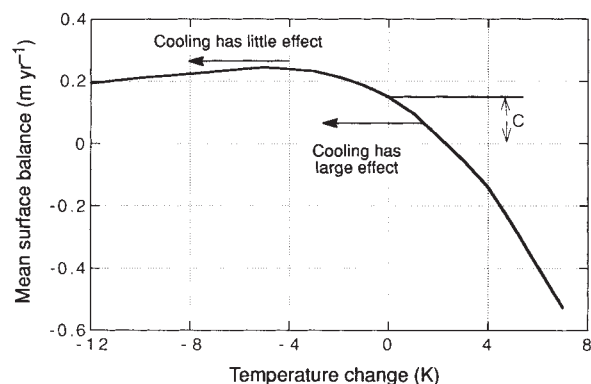


FIG. 4 The surface mass balance (m of water equivalent) of an ice sheet as a function of the change in atmospheric temperature. The calculation is for the Greenland ice sheet in its present state¹¹, but the qualitative shape of the curve is valid for any ice sheet. C is the equivalent amount of calving required for the ice sheet to be in balance. The effect of a cooling on the surface balance, and thus on the calving rate, depends strongly on the climatic regime present.

Orbital parameters are changed every 1,000 yr and the resulting deviation of summer radiation is shown in Fig. 2c, together with the departure of summer temperature. It can be seen that sharp temperature peaks occur shortly after ice-sheet decay, in agreement with the palaeoclimatic record^{21,22}.

Calving and runoff of melt water can be considered separately. The calving rates shown (Fig. 2d) are simply the flux of ice at the edge of the continent, when the ice sheet has reached this. Only sectors 1 and 2 have iceberg discharge. A typical maximum total calving rate is $700 \text{ km}^3 \text{ yr}^{-1}$, which is in very good agreement with estimates from ice-sheet reconstruction¹⁸. For sector 1 the calving rate is approximately constant, whereas more pronounced variations occur in sector 2. Runoff (Fig. 2e) consists of melt water produced on the ice-sheet surface. It is out of phase with iceberg calving. Calving is largest when the ice sheet attains its maximum extent. When decay starts, the runoff becomes larger and larger while calving drops to zero within a few thousand years. It should be noted that typical calving rates are much smaller than typical runoff rates.

Results from the experiment with cooling events can now be compared to the reference case (Fig. 3). Anticipating on an ice-sheet response time of several kyr for this type of limited cooling, a temperature perturbation of -4°C was imposed for 3,000 yr, starting about 3,000 yr before the ^{14}C -age of the Heinrich layers (Fig. 3c). I considered only the last three Heinrich layers, as these are well dated¹ (H1, 14.3 kyr BP; H2, 21 kyr BP; H3: 28 kyr BP—no correction was made to the ^{14}C -age).

The effect on ice volume (not shown) is small. Changes in calving rate for sector 2 are small and negative for H3 and H2, and large and positive for H1. The reason for lower calving rates during the first two cooling events is decreasing accumulation. As can be seen in Fig. 2b, the ice volume in sector 2 is fairly constant between 35,000 and 15,000 yr ago, and so is the calving rate. The imposed cooling events lead to a small perturbation only. For the cooling event starting at 11,300 yr ago the situation is totally different. The ice sheet is in a stage of rapid decay, but the cooling makes the ice sheet stay significantly longer. This then leads to a calving peak at about 15,000 yr ago.

I have run experiments with stronger and longer cooling events imposed. This requires a different tuning to keep the termination in the simulated ice-volume curve. The longer and stronger the cooling, the more the value of f_{ice} has to be reduced. Although stronger cooling events lead to larger changes in calving rates, the picture does not change in a qualitative way.

The results of this study suggest that the Heinrich layers do not result directly from the effect of cooling events on ice-sheet evolution. The effect of cooling depends on the state of the ice sheet and can have either sign. For a large ice sheet in cold conditions, additional cooling brings very little change of iceberg calving rate: it just reduces the accumulation rates slightly (H3 and H2). For a large ice sheet in warm conditions (a situation that cannot last very long), additional cooling reduces the melt rates very strongly (H1). This point is best understood by looking at a detailed mass-balance calculation for an ice sheet of given geometry (Fig. 4). When calculating the mass balance averaged over the entire ice sheet (in this case the Greenland ice sheet¹¹), it becomes clear that the change of mass balance per degree temperature change becomes smaller in colder regimes (and even changes sign). □

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1. Bond, G. et al. *Nature* **360**, 245–249 (1992).
2. Andrews, J. T. & Mahaffy, M. A. *W. Quat. Res.* **6**, 167–183 (1976).
3. Budd, W. F. & Smith, I. N. *Sea Level, Ice, and Climatic Change* (Proc. of the Canberra Symp., Dec. 1979) IAHS Publ. No. 131, 369–409 (1981).
4. Oerlemans, J. *Nature* **287**, 430–432 (1980).
5. Pollard, D. *Nature* **296**, 334–338 (1982).
6. Oerlemans, J. *Clim. Change* **4**, 353–374 (1982).
7. Hyde, W. T. & Peltier, W. R. *J. Atmos. Sci.* **44**, 1351–1374 (1985).
8. Deblonde, G. & Peltier, W. R. *J. geophys. Res.* **96**, 9189–9215 (1991).
9. Gallée, H., Van Ypersele, J. P., Fichefet, T., Tricot, C. & Berger, A. *J. geophys. Res.* **96** (D7), 13139–13161 (1991).
10. Oerlemans, J. & Hoogendoorn, N. C. *J. Glaciol.* **35**, 399–405 (1989).

11. Oerlemans, J. *The Holocene* **1**, 40–49 (1991).
12. Oerlemans, J. & Fortuin, J. P. F. *Science* **258**, 115–117 (1992).
13. Broccoli, A. J. & Manabe, S. *Clim. Dynamics* **1**, 87–99 (1987).
14. Lorius, C., Jouzel, J., Raynaud, D., Hansen, J. & Le Treut, H. *Nature* **347**, 139–145 (1990).
15. Reeh, N. & Gundestrup, N. S. *J. Glaciol.* **31**, 198–200 (1985).
16. Fortuin, J. P. F. *The Surface Mass Balance and Temperature of Antarctica* (Univ. of Utrecht, The Netherlands, 1992).
17. Manabe, S. & Broccoli, A. J. *J. geophys. Res.* **90** (C2), 2167–2190 (1985).
18. Sugden, D. E. *Arctic Alp. Res.* **9**, 21–47 (1977).
19. Denton, G. H. & Hughes, D. J. *The Last Great Ice Sheets* (Wiley-Interscience, New York, 1981).
20. Fisher, D. A., Reeh, N. & Langley, K. *Geogr. phys. Quat.* **39**, 229–238 (1985).
21. Koerner, R. M. *Nature* **343**, 630–631 (1980).
22. Anderson, P. M. et al. (COHMAP Members) *Science* **241**, 1043–1052 (1988).

Past sedimentary organic matter accumulation and degradation controlled by productivity

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CHANGES in bottom-water oxygen concentration are commonly invoked to account for past changes in sedimentary organic carbon accumulation, as organic matter is more readily preserved in anoxic conditions^{1–5}. On the other hand, upper-water productivity has been shown to be the main controlling factor for accumulation of organic matter in modern sediments^{6,7}. It is therefore important to understand whether productivity significantly influenced accumulation patterns in the past, for example during the formation of petroleum source rocks. Here we present a study of a 150-Myr-old sequence containing short-term cycles in organic carbon content from the Kimmeridge Clay formation in Northern England. The lack of bioturbation and the fine-scale laminations throughout this section demonstrate that the sequence was deposited under continuously anoxic bottom-water conditions, so that oxic excursions cannot account for the cyclic accumulation pattern. We show that in this cycle, increased planktonic productivity appears to have caused both increased sedimentary accumulation of refractory organic material, and increased anaerobic degradation of metabolizable organic material. Thus, upper-water productivity should be considered as an important factor in controlling past as well as present accumulation patterns.

The Kimmeridge Clay formation is well known as a lateral equivalent of the major oil source rock unit of the North Sea, outcropping in the Cleveland basin near Marton (England). It consists of alternating organic-rich shales and marls deposited in marginal shallow water under low energy conditions, and reveals a short-term cyclic distribution of the organic content⁸. These cycles (1 m thick on average) have been found to represent 30,000 years⁸, which was evaluated by dividing the duration of an ammonite zone by the number of the cycles within it. One of these microcycles has been investigated by high-resolution sampling (90 samples distributed along an 120-cm-long core section). The total organic carbon content (TOC) values range between 1.8 and 9.5 weight % with the lowest values at the beginning of the cycle and the highest ones in the middle part (Fig. 1).

The TOC variations cannot be explained by a varying dilution by detrital (clay and quartz) and biogenic (mainly coccoliths, occasional diatoms) minerals⁹. Indeed, except for the biogenic

carbonate content, the mineralogical and geochemical composition of the inorganic fraction of the sediment is fairly constant throughout the section, suggesting that variations of the detrital input were weak during the cycle. The carbonate content variations are not strong enough to explain TOC variations and, moreover, are not in phase with them. A diagenetic removal of minerals is also unlikely because no traces of dissolution have been observed. The sediment is fine-grained, stratified with fine-scale laminations, contains abundant sulphides, and does not exhibit any bioturbation. This last character indicates clearly that not only the sediment but also the bottom water has always been anoxic during the cycle. Indeed, in such marginal shallow waters with a high input of organic matter, the absence of heterotrophic and oxygen-dependent benthic life can be explained only by the occurrence of totally anoxic bottom waters over a significant time period. On the other hand, the fine-grained and stratified character of the sediment indicates low energy conditions which permit the development of anoxic bottom conditions when oxygen is totally consumed. The constancy of anoxic conditions are additionally indicated by inorganic markers such as the high vanadium content⁹. Molecular and petrographic investigations^{10,11} have revealed that the origin of the organic matter is mainly phytoplanktonic. Therefore, as the TOC variations cannot be explained by dilution or by changes in redox conditions, they are most plausibly related to varying productivity-related organic fluxes. Moreover, the productivity variations are probably mainly due to mineral-free phytoplankton because TOC and carbonate contents (from coccoliths) variations appear to be independent.

As a consequence of bacterial sulphate reduction, reduced sulphur is often concentrated in organic-rich marine sediments. In our cyclic sequence, most of the sulphur is diagenetically trapped (both as iron sulphides and as organic sulphur compounds) and can therefore be used to estimate the fraction of organic carbon that was oxidized by sulphate reduction. In order

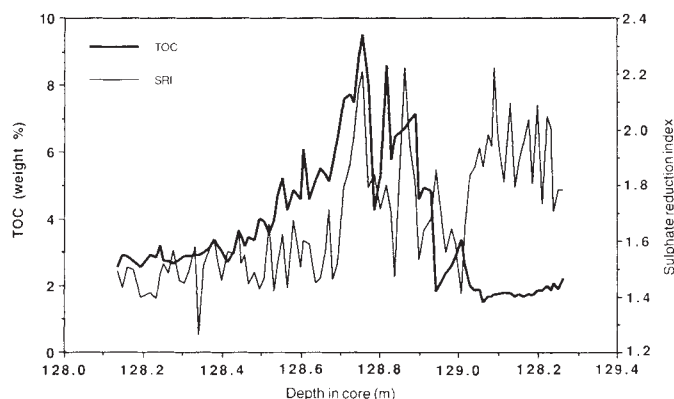


FIG. 1 Variations of total organic carbon content (TOC, in weight %) and sulphate reduction index (SRI) in the deposition cycle. SRI represents the intensity of sulphate reduction relative to the TOC. It is defined as the ratio of (organic carbon preserved + mineralized by sulphate reduction) to preserved organic carbon. Mineralized organic carbon is calculated by using the measured sulphur content and assuming the mean stoichiometry for sulphate reduction¹⁷ $2\text{CH}_2\text{O} + \text{SO}_4^{2-} \rightarrow \text{H}_2\text{S} + 2\text{HCO}_3^-$. We can write $C_{ox} \geq 0.75 S$ where C_{ox} and S denote masses of organic carbon oxidized by sulphate reduction, and reduced sulphur trapped in the sediment respectively. $C_{ox} = 0.75 S$ if sulphur was trapped with perfect efficiency (100%), $C_{ox} = 7.5 S$ if it was trapped with a low efficiency (10%)¹⁷. Some simple mathematical considerations show that the SRI, which is normally a mass or a flux ratio, can reasonably be approached by using the organic carbon and sulphur contents in the same manner. In this study, a mean of 30% of the sulphur (as H_2S produced by sulphate reduction) was assumed to be trapped in the sediment, according to $\delta^{34}\text{S}$ investigations. Comments about SRI variations are given in the text as well as the Fig. 2 legend.

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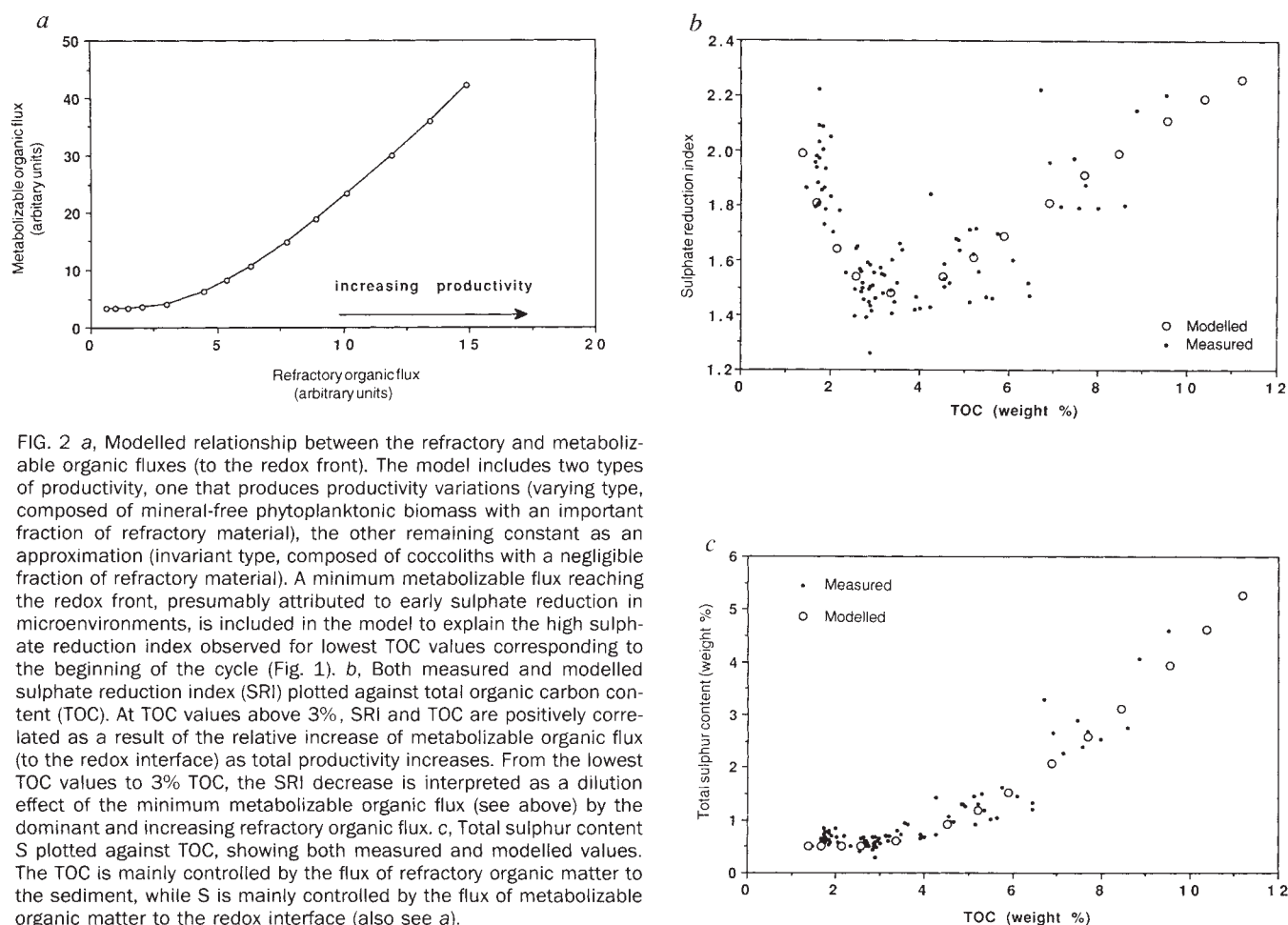


FIG. 2 *a*, Modelled relationship between the refractory and metabolizable organic fluxes (to the redox front). The model includes two types of productivity, one that produces productivity variations (varying type, composed of mineral-free phytoplanktonic biomass with an important fraction of refractory material), the other remaining constant as an approximation (invariant type, composed of coccoliths with a negligible fraction of refractory material). A minimum metabolizable flux reaching the redox front, presumably attributed to early sulphate reduction in microenvironments, is included in the model to explain the high sulphate reduction index observed for lowest TOC values corresponding to the beginning of the cycle (Fig. 1). *b*, Both measured and modelled sulphate reduction index (SRI) plotted against total organic carbon content (TOC). At TOC values above 3%, SRI and TOC are positively correlated as a result of the relative increase of metabolizable organic flux (to the redox interface) as total productivity increases. From the lowest TOC values to 3% TOC, the SRI decrease is interpreted as a dilution effect of the minimum metabolizable organic flux (see above) by the dominant and increasing refractory organic flux. *c*, Total sulphur content *S* plotted against TOC, showing both measured and modelled values. The TOC is mainly controlled by the flux of refractory organic matter to the sediment, while *S* is mainly controlled by the flux of metabolizable organic matter to the redox interface (also see *a*).

to assess fully the organic carbon oxidized by sulphate reduction it is necessary to evaluate the efficiency with which H_2S has been retained in the sediment, or, at least, to speculate on it according to the values generally given in the literature. By using the 'sulphate reduction index' (SRI) calculated from TOC and total sulphur content¹², it is possible to evaluate the sulphate reduction intensity relative to the preserved organic carbon flux. The variations of SRI and TOC in the cycle (Fig. 1) show that changes in the productivity-related organic carbon flux have been accompanied by variations in the sulphate reduction intensity. Some of the highest SRI values are associated with the highest organic carbon contents. This is interpreted as the result of an increasing metabolizable organic flux (which is totally remineralized by sulphate reduction), combined with an increasing refractory organic flux as the productivity increases. As a result of the position of the redox front above the water-sediment interface, it is assumed that dissolved sulphate was not a limiting factor for sulphate reduction. Such an interpretation is in agreement with biological models of organic exportation from the photic zone to the sediment¹³, and with the evidence that the degradation of organic matter by aerobes and anaerobes (mainly sulphate-reducers in marine environments) is very similar where the supply of metabolizable organic matter is similar¹⁴. The relationship between TOC and the total sulphur content can thus be regarded as a reflection of the relative variations of the metabolizable flux and the refractory flux (Fig. 2*a* and *c*). It indicates that, during a single short-term depositional cycle, the metabolizable/refractory ratio in the exported organic carbon flux (to the redox interface) increases with productivity up to a constant limit that corresponds to the slope of the quasi-linear part of the

curve. Presumably, this limit depends on both the metabolizable/refractory ratio in the living biomass and the distance between the euphotic zone and the redox interface.

Because we observe that more organic carbon was metabolized when a larger accumulation of organic carbon took place, the major mechanism for accumulation should be a selective preservation of biologically resistant organic matter from plankton. Indeed, this is confirmed by the presence of laminar and ultralaminar biologically resistant material observed by transmission electron microscopy, similar to that reported by Largeau *et al.*¹⁵.

These interpretations are in agreement with those of Calvert and Pedersen⁷ concerning the dominant control by the primary production in the overlying water column, and the minor control by the redox conditions in deep water in organic preservation. Indeed, the selective preservation of refractory organic matter from planktonic productivity is a mechanism which can occur either in oxic or anoxic conditions, and the accumulation of organic carbon is mainly linked to the flux of this material relative to the bulk flux. Variations in redox conditions play a role only in changing the degradation of metabolizable organic carbon from an aerobic pathway using oxygen (respiration of macro- and micro-organisms, and direct chemical oxidation) to an anaerobic bacterial pathway using other oxidants such as nitrate, sulphate, oxy-hydroxides of manganese and iron, or organic matter itself (fermentative bacterial activity).

High SRI values are also observed in association with the lowest TOC (2%) at the beginning of the cycle, and result from total sulphur contents of ~0.5–0.7%. This sulphur was recognized as being derived from sulphate reduction because of the

presence of pyrite framboids, but is associated with a different kind of planktonic organic carbon. This is revealed by a different texture and colour of the amorphous organic matter in palynofacies, as well as by the different proportions of laminar and ultralaminar material found by scanning transmission electron microscopy. The interpretation of these high SRI values is still speculative. It could be due to a larger relative influence of coccolith productivity modifying conditions for export from the photic zone (more efficient protection by the mineral tests against degradation). More probably, the cause could be early sulphate reduction in anaerobic microenvironments (fecal pellets, aggregates, living or dead organisms) close to the photic zone (where metabolizable organic matter is still available even in low or moderate productivity conditions).

Our interpretations of the role of productivity as a primary factor in sediment composition, and of a selective accumulation pathway through sulphate reduction, can be integrated into a mass-balance model. This model (P.B. *et al.*, manuscript in preparation) simulates a sediment composition from initial fluxes (mineral and organic fluxes from both planktonic productivity and detrital input). Productivity variations of mineral-free phytoplankton are imposed on the model by means of varying fluxes of refractory organic matter, whereas the productivity variations of mineral-bearing phytoplankton (mainly coccoliths) are considered to be negligible according to dilution considerations which have been previously mentioned. The metabolizable organic matter flux is calculated as a function of the refractory organic matter flux in such a way that metabolizable/refractory ratio increases as the productivity increases, as indicated in Fig. 2a. When productivity is still moderate (1–5% TOC), the organic flux to the redox interface is mainly composed of refractory organic matter because the metabolizable material is highly recycled in the photic zone, and thus the refractory flux increases more rapidly than the metabolizable one. As productivity increases, the export efficiency of the metabolizable organic carbon increases due to modifications in the biosedimentary processes up to a constant limit, expressed in the linear relationship between both fluxes. This limit is thought to depend on the type of varying productivity (metabolizable/refractory organic carbon in the living biomass) and on the local conditions (distance between the photic zone and the redox interface). The detrital flux variations are assumed to be negligible relative to the organic flux variations from planktonic productivity. The invariant biogenic and detrital mineral fluxes are adjusted in such a way that they give a correct simulation of sediment composition when productivity is lowest. Sulphate reduction is assumed to be the dominant degradation process of organic matter beyond the redox front, to the extent that the sulphate access is not limited. This degradation is therefore only limited by the flux of the metabolizable organic matter.

As shown in Fig. 2b and c, the modelling results can be adjusted to the measured ones. In turn, this accordance between the modelled and measured results further supports our interpretations and assumptions. However, the validity of the model will have to be verified on modern equivalent sedimentary records from which real and absolute fluxes can be directly measured with the aid of high-resolution chronostratigraphy. This may lead to new ways of relating the preserved organic carbon to palaeoproductivity variations throughout glacial-interglacial successions.

Continuing studies show that, in cycles with very high TOC (up to 35%), the sulphate reduction intensity apparently decreases as TOC increases. Such observations were found both in the Kimmeridge Clay formation and in the Kimmeridgian shales of Dorset^{12,16}, and are interpreted as the result of restricted sulphate reduction. In such a case, a significant fraction of the metabolizable organic matter is rapidly buried due to a high sedimentation rate which is mainly controlled by the organic flux. The higher the organic flux, the lower the free access to seawater sulphate. The subsequent behaviour of the rapidly-

buried metabolizable organic matter (either degradation by fermentative bacterial activities or preservation) should be elucidated and modelled. □

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1. Demaison, G. J. & Moore, G. T. *Am. Ass. Petrol. Geologists Bull.* **64**, 1179–1180 (1980).
2. de Graciansky, P. C. *et al. Nature* **308**, 346–349 (1984).
3. Stow, D. A. V. & Dean, W. E. *Init. Rep. DSDP Leg 75*, 805–818 (1984).
4. Schiøt, S. O. & Jenkins, H. C. *Geologie Mijnb.* **55**, 179–184 (1976).
5. Thiede, J. & van Andel, T. J. *Earth planet. Sci. Lett.* **33**, 301–309 (1977).
6. Pedersen, T. F. & Calvert, S. E. *Am. Ass. Petrol. Geologists Bull.* **74**, 454–466 (1990).
7. Calvert, S. E. & Pedersen, T. F. in *Organic Matter: Productivity, Accumulation, and Preservation in Recent and Ancient Sediments* (eds Whelan, J. K. & Farrington, J. W.) 231–263 (Columbia Univ. Press, New York, 1992).
8. Herbin, J.-P., Müller, C., Geyssant, J. R., Mélières, F. & Penn, I. E. *Rev. inst. Fr. Pét.* **46**, 675–712 (1991).
9. Tribouillard, N. *et al. C.R. hebdom. Séanc. Acad. Sci., Paris, Série II*, **314**, 923–930 (1992).
10. Ramanampisoa, L. *et al. C.R. hebdom. Séanc. Acad. Sci., Paris, Série II*, **314**, 1493–1498 (1992).
11. Pradier, B. & Bertrand, P. *C.R. hebdom. Séanc. Acad. Sci., Paris, Série II*, **315**, 187–192 (1992).
12. Lallier-Vergès, E., Bertrand, P., Huc, A. Y., Büchel, D. & Tremblay, P. *Marine and Petroleum Geology* (Elsevier, Amsterdam) (in the press).
13. Wefer, G. in *Report of the Dahlem Workshop on Productivity of the Oceans: Present and Past* (eds Berger, W. H., Smetacek, V. S. & Wefer, G.) 139–154 (Wiley, Chichester, 1989).
14. Jørgensen, B. B. *Nature* **296**, 643–645 (1982).
15. Largeau, C. *et al. in Advances in Organic Geochemistry 1989* (eds Durand B. & Behar F.) 889–895 (Pergamon Press, Oxford, 1990).
16. Huc, A. Y., Lallier-Vergès, E., Bertrand, P., Carpentier, B. & Hollander, D. J. in *Organic Matter: Productivity, Accumulation, and Preservation in Recent and Ancient Sediments* (eds Whelan, J. K. & Farrington, J. W.) 469–486 (Columbia Univ. Press, New York, 1992).
17. Berner, R. A. & Raiswell, R. *Geochim. cosmochim. Acta* **47**, 855–862 (1983).

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Confirmation of the astronomical calibration of the magnetic polarity timescale from sea-floor spreading rates

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THE dating of the history of reversals of the Earth's magnetic field since the Jurassic period has been based primarily on radiometric dating of a small number of reversals, with most ages of individual reversals interpolated or extrapolated on the basis of the widths of marine magnetic anomalies. Recently, however, Pliocene and Pleistocene reversals have been dated by an entirely independent method, which relies on correlating climate proxies (such as oxygen isotope ratios) in sediments with calculated variations of the Earth's orbit and inclination^{1–4}. The dates obtained from this astronomical calibration are ~3–8% older than radiometrically calibrated ages published between 1979 and 1989 (see, for example, refs 5 and 6), although recent dating by the ⁴⁰Ar/³⁹Ar method tends to confirm the astronomical calibration^{7–10}, at least for the past 3.3 Myr. Here I use sea-floor spreading rates determined from high-precision plate-rotation solutions for five plate pairs to test the astronomically calibrated timescale over the past 5.32 Myr. The results suggest that the errors in the astronomical calibration are no greater than 0.02 Myr, and also show that spreading rate can remain constant for several million years, even for fast-moving plates.

Constraints from marine anomalies have generally been derived from measurements of the widths of particular polarity intervals on individual profiles, averaging several widths where possible from both sides of the spreading ridge. A significant source of noise is the process of asymmetric spreading, which causes variations in the width of polarity stripes even when the

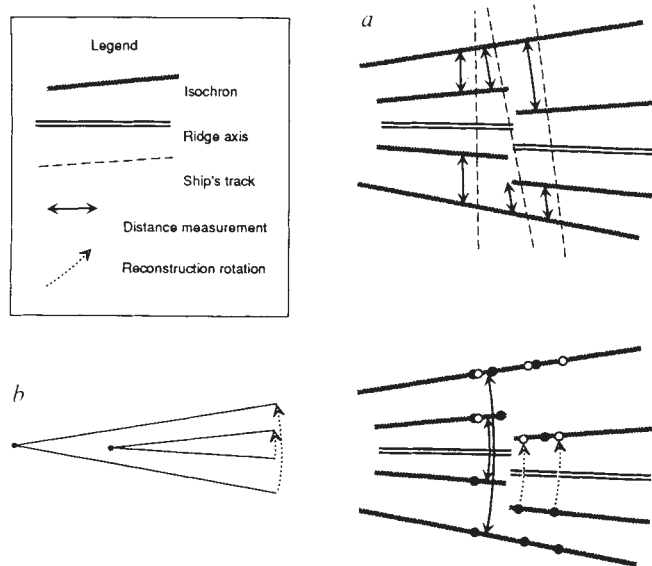


FIG. 1 Schematic diagram illustrating two methods of measuring spreading distance for isochrons identified from marine magnetic anomalies. *a*, Measurement by averaging of anomaly widths is vulnerable to noise introduced by changes in the spreading-ridge geometry, commonly of the order of 5–10 km. *b*, Measurement by determining total-rotation poles by matching isochron observations (solid dots) on opposite plates bypasses this source of noise. At any location, the spreading distance for each isochron can be determined from the distance to the rotation pole and the rotation angle. Precision can be of the order of 1 km.

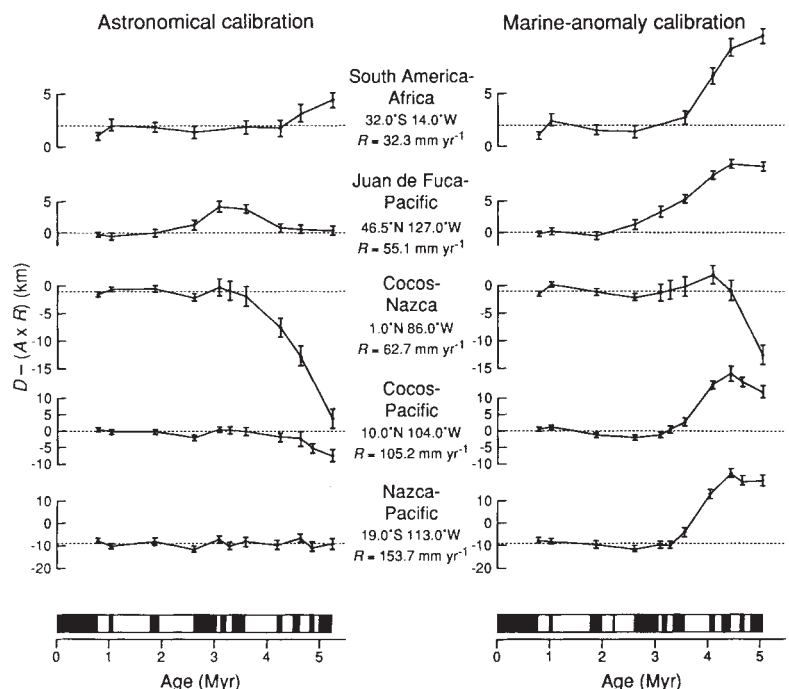
plate motion is constant and the tectonic plates are rigid (Fig. 1). For well-surveyed plate pairs, this source of noise can be avoided by measuring the distance between matching isochrons on opposite plates and determining the distance between successive isochrons by subtraction. Determination of rotation poles provides a statistically rigorous method of measuring the total spreading distance, with uncertainty, of a given isochron. To

interpolate between dated isochrons, one or more plate pairs must be assumed to have constant or smoothly varying spreading rate.

The present analysis uses high-precision plate reconstructions for five plate pairs over the age interval covered by the astronomical calibration of the reversal time scale (0–5.32 Myr). Details of the reconstructions have been or will be reported elsewhere (ref. 11; C. Weiland *et al.*, and D.S.W., manuscripts in preparation); here I concentrate on the spreading distances (with uncertainties) and their implications for the timescale. The location of rotation poles and the associated gradients in spreading rate provide information about the stability of plate motion, independent of time information. Two plate pairs, Pacific–Nazca and Africa–South America, show no evidence for changes in the location of their rotation poles, and the pole for the Pacific–Cocos pair changes only slightly. Motion of the Cocos plate relative to the Nazca plate shows two apparently abrupt changes at 3.5–4 and 1–1.5 Myr, in both cases with the observed westward decrease in spreading rate becoming less pronounced after the change. Motion of the Juan de Fuca plate relative to the Pacific plate changes from a gradient in rate faster to the south (before 3–4 Myr) to an opposite gradient (since 1–2 Myr); it is not possible to resolve the data sufficiently to tell whether several abrupt changes or a gradual change occurred.

Measurements of spreading distance for a plate pair allow a simple test (for any timescale) of the hypothesis of constant spreading rate, which predicts a linear relation between observed distance and isochron age. It is more useful to plot the departure of the observed distance from the distance predicted by a tested constant rate (reduced distance), rather than total observed distance, as reduced distance can be plotted on an expanded scale. Figure 2 shows reduced-distance plots for the five plate pairs and two timescales. The astronomically calibrated scale is assembled from Shackleton *et al.*² (<3 Myr) and Hilgen⁴ (>3 Myr); Cande and Kent¹² give the most recent timescale from marine magnetic anomalies. The scale of Cande and Kent is calibrated to agree with that of Shackleton *et al.* at 0.78 and 2.60 Myr before present, but is 0.15–0.20 Myr younger than the older part of the Hilgen scale. Each distance is based on the length defined by the rotation angle of a small-circle chord centred on the rotation pole (Fig. 1) and passing through the indicated point. The uncer-

FIG. 2 Reduced-distance plots for five plate pairs, contrasting astronomical calibration of the time scale^{2,4} (left) with a recent scale derived from widths of magnetic anomalies¹² (right). Predicted distance based on isochron age (*A*) and indicated full spreading rate (*R*) has been subtracted from the total spreading distance (*D*) measured from rotation-pole solutions by small-circle chords through the indicated locations. Horizontal dotted lines highlight intervals consistent with constant spreading at the reduction rate. Positive slopes indicate faster rates, and negative slopes, slower rates. The vertical distance scales are plotted inversely with the spreading rate so that timescale errors will have the same vertical amplitude for plate pairs with constant motion.



tainty of each distance is found by searching the 95% confidence interval of the total rotation for maximum and minimum spreading distance. Each rotation confidence interval is based on a grid search for a threshold misfit determined by the *F*-ratio technique described by Chang¹³. This operation of projecting the confidence interval for total rotation (3 parameters) into the confidence interval for spreading distance (1 parameter) should result in a confidence level significantly above 95%. For the best-surveyed isochrons, the full width of the confidence interval in spreading distance is about 1 km.

In detail, no plate pair is perfectly consistent with constant spreading rate for either timescale. The rate variations implied by the astronomical calibration are either much smaller or more consistent with physically plausible changes in spreading rate, when compared with Cande and Kent or any other published timescale. The only case where the astronomical calibration implies a larger change in rate is for the Cocos-Nazca plate pair for 3–5 Myr. Both pairs involving the Cocos plate are consistent with a change in rate at about 3.8–4 Myr, in accord with the independently identified change in the location of the rotation pole (D.S.W., manuscript in preparation). Assuming that each reversal age is uncertain by 0.01 Myr, Nazca-Pacific spreading is consistent with a constant rate for the interval 1.0–5.2 Myr, Cocos-Pacific for 0.0–3.6 Myr, Cocos-Nazca for 1.0–3.6 Myr, and Africa-South America for 1.0–4.5 Myr. In contrast, rates implied by the Cande and Kent scale are about 20% faster from 3.5 to 4.5 Myr than from 1.0 to 3.0 Myr for four of five plate pairs. Comparable rate changes are implied by all other timescales descended from the Klitgord *et al.*¹⁴ scale, which was based on the assumption (now seen to be erroneous) that Cocos-Nazca motion was constant.

Minor refinements to the astronomical scale can be inferred from Fig. 2. The age of 2.60 Myr (ref. 2) for C2Ay (Matuyama/Gauss) appears too old by 0.01–0.02 Myr, based on its reduced distance plotting below adjacent isochrons. Similarly, 4.62 Myr (ref. 4) for C3.2o (base of Nunivak) may be too young by a similar margin, although uncertainties are larger. After these adjustments, the maximum permitted rate variations averaged over 1 million years for Cocos-Pacific (0–3.6 Myr) or Nazca-Pacific (1.0–5.2 Myr) are about 3%. The fact that several plate pairs are consistent with constant spreading rate at very high precision according to the astronomical calibration lends strong support to the calibration technique. Rigorous confirmation of the calibration will have to come from accurate radiometric dating of more of the reversals, as nothing reported here contradicts the (implausible) alternative hypothesis that synchronous changes in plate motion have exactly the same magnitude as errors in the dating. It is so unusual to find datable material of an age within 0.02 Myr of a reversal that only Brunhes/Matuyama (0.78 Myr) is radiometrically dated to that precision^{7,8}. The assumption that multiple plate pairs do not have simultaneous rate changes of the same magnitude implies that none of the errors in the astronomical calibration exceed 0.02 Myr. The implications of a revised timescale that is both precise and accurate are obviously broad. One example is that the plate-motion rates of DeMets *et al.*¹⁵ need to be revised downward by 4–5%.

The observation that Nazca-Pacific motion was constant for most of the calibrated time interval is also significant. Cande and Kent¹² and many previous workers used few constraints from the faster plates, assuming that plates with significant subducting slabs are more prone to changes in motion. Perhaps plate size is a better predictor of stability. The smaller Juan de Fuca and Cocos plates have fairly unstable motion, whereas both the Nazca-Pacific and Africa-South America pairs show relative stability. Both fast and slow plates seem well described by intervals of constant spreading rate separated by unresolvably brief changes in motion. These patterns are consistent with generally accepted models (for example, ref. 16) that plate motion is driven primarily by body forces generated by the high

density of the plates relative to the asthenosphere. If plate-motion changes are caused by changes in the boundary geometry, a given change will be more likely to measurably affect the motion of a small plate than a large one. The possibility of constant motion of fast plates offers an opportunity for high-resolution calibration of the part of the timescale too old for astronomical calibration. □

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1. Johnson, R. G. *Quat. Res.* **17**, 135–147 (1982).
2. Shackleton, N. J., Berger, A. & Peltier, W. R. *Trans. R. Soc. Edinb.* **81**, 251–261 (1990).
3. Hilgen, F. J. *Earth planet. Sci. Lett.* **104**, 226–244 (1991).
4. Hilgen, F. J. *Earth planet. Sci. Lett.* **107**, 349–368 (1991).
5. Mankinen, E. A. & Dalrymple, G. B. *J. geophys. Res.* **84**, 615–626 (1979).
6. Harland, W. B. *et al.* *A Geologic Time Scale 1989* (Cambridge Univ. Press, 1990).
7. Baksi, A. K., Hsu, V., McWilliams, M. O. & Farrar, E. *Science* **256**, 356–357 (1992).
8. Spell, T. L. & McDougall, I. *Geophys. Res. Lett.* **19**, 1181–1184 (1992).
9. Tauxe, L., Deino, A. D., Behrensmeier, A. K. & Potts, R. *Earth planet. Sci. Lett.* **109**, 561–572 (1992).
10. McDougall, I., Brown, F. H., Cerling, T. E. & Hillhouse, J. W. *Geophys. Res. Lett.* **19**, 2349–2352 (1992).
11. Wilson, D. S. *J. geophys. Res.* (in the press).
12. Cande, S. C. & Kent, D. V. *J. geophys. Res.* **97**, 13917–13951 (1992).
13. Chang, T. J. *Am. statist. Ass.* **83**, 1178–1183 (1988).
14. Klitgord, K. D., Huestis, S. P., Mudie, J. D. & Parker, R. L. *Geophys. J. R. astr. Soc.* **43**, 387–424 (1975).
15. DeMets, C., Gordon, R. G., Argus, D. F. & Stein, S. *Geophys. J. Int.* **101**, 425–478 (1990).
16. Forsyth, D. & Uyeda, S. *Geophys. J. R. astr. Soc.* **43**, 163–200 (1975).

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Evidence for transformational faulting from a deep double seismic zone in Tonga

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DOUBLE seismic zones, planes or earthquakes parallel to the dip of a subducting slab and separated by 20–40 km, provide important clues about the earthquake generating mechanisms and strain distribution inside subducting slabs. Double seismic zones have been found at intermediate depths (70–200 km) in many subduction zones^{1–6} but have not been previously reported in deep slabs. Here, by relocating earthquakes with a hypocentroidal decomposition technique⁷ and visualizing the earthquake positions and uncertainties in three dimensions, we identify a double seismic zone at depths of 350–460 km in the Tonga subduction zone. Source parameters of the earthquakes determined by waveform analysis suggest different stress orientations for the two zones, with in-plane compression in the lower zone and in-plane tension in the upper zone. The double zone may be due to transformational faulting, as olivine along the edges of a metastable olivine wedge becomes warmer and transforms to spinel^{8–11}.

The spatial configuration of deep earthquakes is one of the primary constraints on deep-slab processes. We determine the relative positions and uncertainties of earthquakes within a region of the slab by inverting P, PKP and pP arrival times from the International Seismological Center listings (1964–1990) using a hypocentroidal decomposition algorithm⁷. This method uses arrival times from different earthquakes at the same station to constrain earthquake relative positions within a limited source region, thus minimizing the effect of unknown velocity heterogeneity along the ray paths. The station set need not be the same for all events, as this would eliminate most of the data, but any station that records two or more of the events is used as a constraint on the relative positions of the corresponding events.

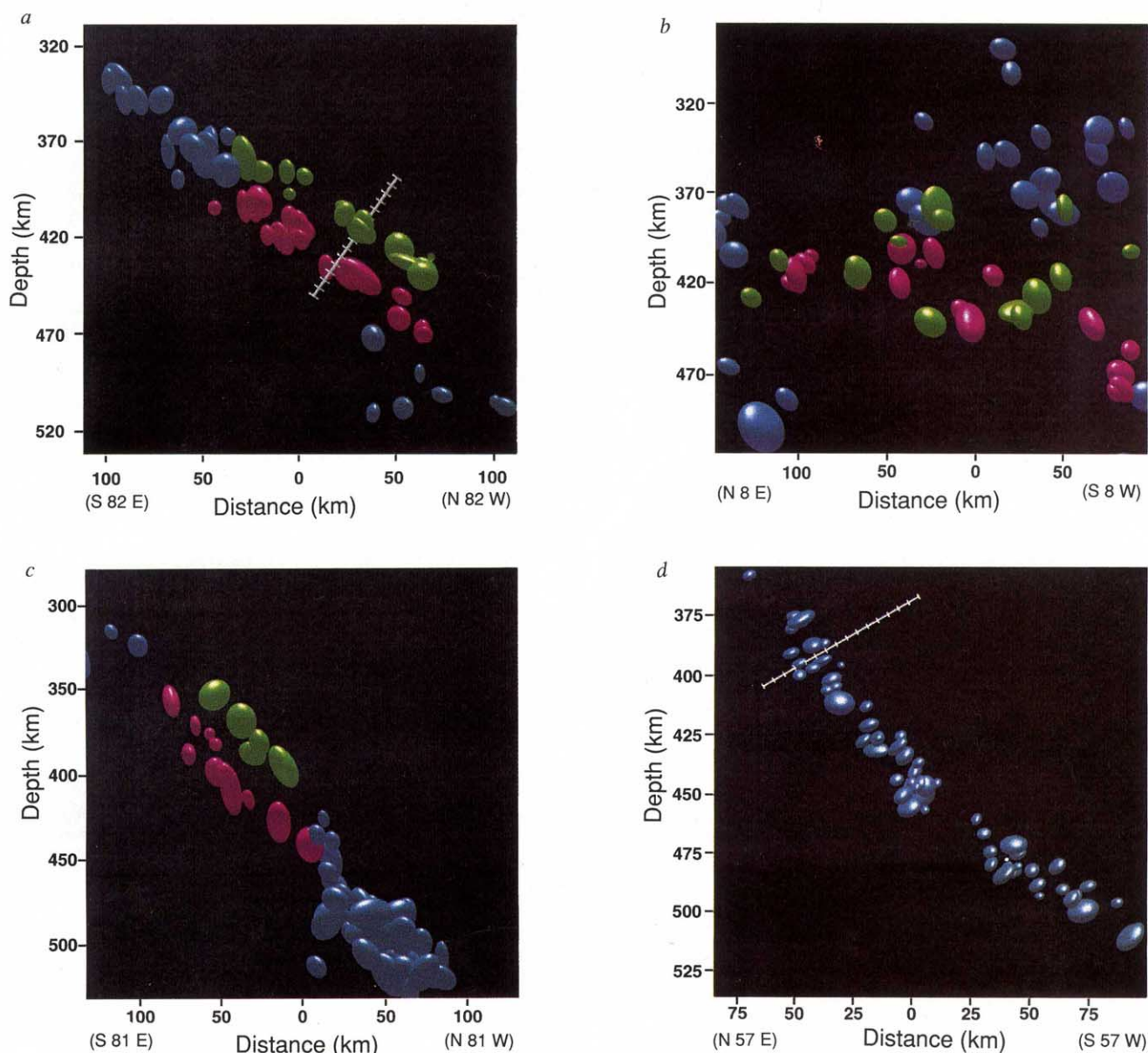


FIG. 1 Three-dimensional views of the Tonga and Izu-Bonin slabs. Each figure shows the 95% confidence ellipsoid determined by a hypocentral decomposition inversion⁷ for each earthquake. *a*, Along-strike view of the double seismic zone in north Tonga. Earthquake positions were determined by inversion of 8,900 arrival times for the locations of 103 events. Earthquakes in the upper zone are denoted by a light green colour; events in the lower zone by red; and events not clearly associated with either zone by blue. The double zone is clearly delineated by the separation of the uncertainty ellipsoids. The scatter of earthquakes below the double zone results from different slab dips in the front and back regions of the view. A 'ruler' with a tick-mark spacing of 5 km demonstrates that the separation of the centre of the zones is ~ 25 km. View is from the NNE and centred on 17.5° S, 177.4° W, with

a depth of field of 200 km along the arc. *b*, View of the northern zone from the WNW, perpendicular to Fig. 1*a* and to the slab strike, showing that in projection the upper-zone earthquakes overlap the lower-zone events and do not represent segments of the slab with different dips. Other parameters the same as *a*. *c*, Along-strike view of the double seismic zone in south Tonga. 8,625 arrival times were inverted for the positions of 116 earthquakes. The view is centred on 24° S, 179.3° W and the depth of field is 240 km. *d*, Along-strike view of the Izu-Bonin slab, where no double zone is found. 14,450 arrival times were inverted for the positions of 108 earthquakes. Ruler with tick mark spacing of 5 km demonstrates that the seismic zone is at most 15–20 km wide. View is from the NNW and centred at 30.8° N, 138.2° E, with a depth of field of 300 km.

The region encompassed by a relocation is typically limited to several hundred kilometres in length and depth to ensure that the rays from each earthquake encounter similar velocity heterogeneity between the source and receiver. Only events with more than 30 arrival times are inverted, and events showing a 95% confidence semi-axis length of greater than 15 km are eliminated from the final inversion.

Relocation of earthquakes between depths of 300 and 500 km in Tonga reveals two planes of seismicity (Fig. 1*a, c*) in the

northern and southern regions of the slab (16.5 – 18.5° S and 23.0 – 25° S); there is a suggestion of double zone in central Tonga, but this is less well defined. The northern double zone occurs between depths of 380 and 460 km for an along-arc distance of about 200 km. Views along the strike of the slab show that the 95% confidence regions of the earthquakes in the two planes are well separated (Fig. 1*a*). A view perpendicular to the slab strike (Fig. 1*b*) shows that the upper zone earthquakes overlie the lower zone. We determined the best planar surface which

TABLE 1 Focal mechanisms for Tonga double zone earthquakes

Year	Month	Day	Lat.*	Long.	Depth	Origin time	Strike	Dip	Slip	m_b	Mo†	Source‡	Region§
1975	2	2	17.097	177.290	384.9	15:51:22.4	25	75	15	5.2	6.7	TS	NUZ
1985	11	05	16.568	177.486	407.6	0:53:17.4	00	20	240	5.1	0.6	TS	NUZ
1987	11	12	17.163	177.312	396.4	0:24:41.8	287	30	-114	5.4	6.0	CMT	NUZ
1988	6	19	18.238	177.755	401.4	13:05:05.7	32	47	36	5.2	2.0	CMT	NUZ
1978	11	18	16.760	176.937	400.3	15:32:58.0	270	47	40	5.3	5.80	CMT	NLZ
1984	4	25	17.288	177.160	408.6	4:19:32.6	277	16	-4	5.6	30.0	CMT	NLZ
1984	9	1	18.137	178.140	472.4	21:16:09.6	02	52	-12	5.3	3.5	CMT	NLZ
1971	5	26	24.888	179.107	412.0	00:11:56.1	25	85	270	5.4	15.0	TS	SLZ
1973	7	21	24.889	179.122	414.6	04:19:18.5	91	55	-167	5.9	NA	RI	SLZ
1975	2	22	25.086	178.808	365.6	22:04:37.3	118	17	-143	6.1	NA	RI	SLZ
1981	10	24	24.841	178.953	396.3	04:57:55.5	131	43	173	5.1	0.69	CMT	SLZ
1986	3	28	23.007	178.663	389.7	02:56:28.9	152	38	-148	5.2	3.7	CMT	SLZ

* Relocated locations given in latitude (degrees south), longitude (degrees west) and depth (km).

† Seismic moment in units of 10^{17} N m.

‡ References for focal mechanisms: this study (TS), the Harvard centroid moment tensor data set (CMT) and a previous study (RI, ref. 31).

§ Region: the upper zone of the northern double zone (NUZ), the lower zone of the northern double zone (NLZ) and the lower zone of the southern double seismic zone (SLZ).

would fit each of the two zones of earthquakes using a least-squares fitting program. The results show that the zones form nearly parallel planes with a consistent separation of 25 km.

The southern double zone occurs between depths of 350 km and 420 km. The upper zone is less seismically active in the south, comprising only five earthquakes. However, the confidence regions of the upper and lower zones are well separated (Fig. 1c), and when projected onto a single plane parallel to the slab, the upper zone earthquakes overlap those in the lower zone (Fig. 1b). The Tonga slab contrasts with the appearance of other subducting slabs at depths of 300–500 km, which generally show a simple single plane of earthquakes with a width of 15–25 km, as illustrated by the Izu-Bonin slab (Fig. 1d).

Both northern and southern double zones are robust features, and are present regardless of whether pP and PKP phases are included in the inversion. The most poorly constrained parameter is generally source depth, and the best depth constraints come from either nearby stations or pP phase observations where

the phases take off upward from the source. 43 of the 49 events in the double zones have two or more observations of this type, and there is no systematic difference in the quality of the depth constraints between the events in the two zones. There is also no systematic presence or absence of any highly important local station in either the upper or lower zone. Events in both upper and lower zones occur throughout the time period of the dataset.

We used earthquake focal mechanisms to investigate the state of stress in the double zone. Focal mechanisms were determined by a waveform-inversion method, which matches the teleseismic body wavetrain using synthetics calculated with a reflective method^{12,13}. The results (Table 1), combined with mechanisms from previous studies, suggest that the lower planes in both northern and southern regions are characterized by compressive stress in the plane of the slab (Fig. 2). The upper zone in the northern region shows a range of faulting types, but the mechanisms generally show tension in the plane of the slab.

The 400-km-deep double zone is not a continuation of the

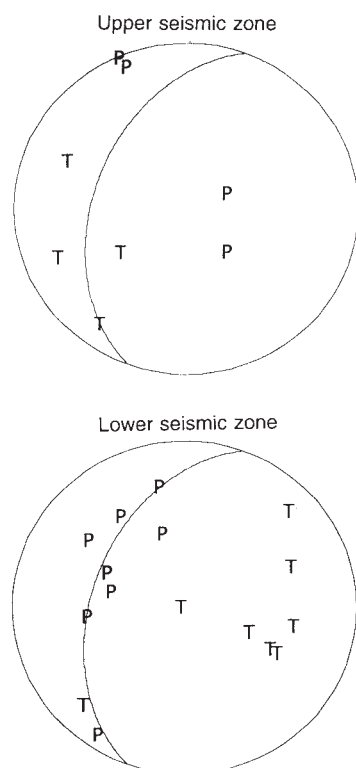


FIG. 2 Stereonet plots of the compressional (P) and tensional (T) axes of earthquakes in the upper- and lower-planes of the Tonga double seismic zones. Earthquake source parameters (listed in Table 1) are taken from this study, previous work³¹ and from Harvard centroid moment tensor solutions³². All upper-zone earthquakes with focal mechanisms are located in the northern region, as events in the southern upper zone are too small for mechanism determination. The lower zone plot contains events from both northern and southern regions. A plane representing the slab orientation is also plotted. Axes from the upper zone are scattered, but generally indicate tension within the plane of the slab, whereas axes from the lower zone indicate compression in the plane of the slab.

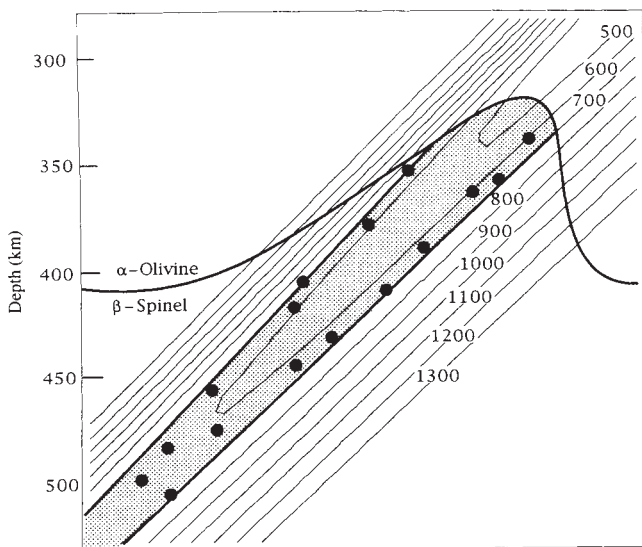


FIG. 3 Finite difference thermal model of the Tonga slab, with temperature contours given in °C. The elevation of the olivine to β -spinel transition is shown, calculated using a compilation of thermodynamic data²⁷. Other thermodynamic datasets show a similar elevation of the olivine to olivine + γ -spinel transition²⁸. The metastable olivine wedge is stippled, assuming that the reaction is kinetically inhibited below 700 °C^{21,22}. Transformational earthquakes associated with the metastable transition should occur in the warmer regions of the metastable wedge, forming lanes of seismicity along the upper and lower boundaries between metastable olivine and spinel. Earthquakes are shown schematically along these zones, which begin at depths of about 350 km and converge at greater depths.

intermediate-depth double zone in Tonga^{5,14}. The stress pattern of the deeper double zone, with in-plane tension in the upper plane and compression in the lower plane, is opposite that of the intermediate-depth double zone^{5,14}. The intermediate double zone seems absent at depths greater than 250 km, although the seismic zone is not well resolved at 250–350 km depth due to the low seismicity rate. The deep double zone is also well separated from a double slab at the base of the subduction zone, where seismicity is displaced 90 km to the west¹⁵.

The deep double seismic zone in Tonga may be caused by bending stresses associated with the increase in dip angle of the Tonga slab just below the double zone. Stresses in the double zone, with the upper region under in-plane tension and the lower region under compression, are consistent with this interpretation, and intermediate-depth double zones are often attributed to bending or unbending of the slab^{2,4,16–18}. The seismic zone is fairly planar however, and is not undergoing obvious bending in the region of the double zone itself (Fig. 1a and c).

A more intriguing possibility is that the deep double seismic zone may provide evidence that deep earthquakes occur through transformational faulting. Although the location of the thermodynamically predicted olivine to spinel phase transition is elevated in the cold slab^{19,20}, the transition should be kinetically delayed at temperatures below about 700 °C^{21,22}, as found in the coldest parts of old, rapidly subducting slabs. Experimental evidence^{8–11} suggests that the transition from metastable olivine to spinel may be accompanied by shear dislocations, an explanation for deep earthquakes that is compatible with changes in the depth distribution^{23,24} and rise times²⁵ of earthquakes at depths of 300–400 km.

Thermal modelling of the Tonga slab using a finite difference method²⁶ suggests that the metastable region should be about 30 km wide at a depth of 400 km and have a temperature near 500 °C at its centre (Fig. 3). Under these conditions, transformational faulting should occur preferentially along the warmer edges of the metastable wedge at temperatures of ~700 °C rather

than at the cold core of the slab. The transformational earthquakes would thus form a double seismic zone, beginning near the depth where the 700 °C isotherm intersects the thermodynamically expected phase transformation. At greater depths, the core of the metastable wedge warms, and transformational faulting should occur throughout the wedge.

There is some uncertainty concerning the olivine phase relations at low temperatures in the slab. In our thermal model, either the olivine to β -spinel^{19,27} or the olivine to olivine + γ -spinel²⁸ phase transition is elevated to nearly 300 km depth in the centre of the slab, and intersects the 700 °C isotherm at depths of 330–350 km (Fig. 3). Thus transformational faulting should produce two parallel bands of seismicity separated by ~25–30 km and beginning at depths somewhat greater than 300 km, in accord with the observed double seismic zone in Tonga.

Simple stress models for slabs predict large in-plane tensional stresses along the top interface of the metastable wedge, with large compressional stresses in the central core and smaller tensional stresses at the low wedge interface²⁹. This is in agreement with the observed tensional stress orientation in the upper zone, but the lower tensional zone is not observed in Tonga. Compressional stresses observed along the lower interface may be explained by a component of compressional tectonic stress from resistance to slab penetration at depth. Alternatively, the lower zone may represent the central compressive core of the metastable wedge, with earthquakes localized preferentially as transformational faulting is activated by large deviatoric stresses¹⁰. This would imply that stress is more important than temperature in activating the metastable transition, and that the lower interface of the metastable wedge is aseismic.

It is difficult to explain the absence of double zones in other deep subduction zones if either bending or metastable transitions are used to explain the Tonga double zone. For example, a double zone is not present in the deep Izu-Bonin slab despite the bending of the slab (Fig. 1d). One possibility is that the double zone in Tonga results from the very high subduction velocity of the Tonga slab³⁰, which would produce lower temperatures in the core of the slab and facilitate the production of a double seismic zone by transformational faulting. □

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- Umino, N. & Hasegawa, A. *J. Seismol. Soc. Jap. Ser. 2* **28**, 125–139 (1975).
- Isacks, B. L. & Barazangi, M. *Geometry of the Beniof Zones: Lateral Segmentation and Downwards Bending of the Subducted Lithosphere* Vol. 1, 99–114 (Am. Geophys. Un., Washington DC, 1977).
- Hasegawa, A., Umino, N. & Takagi, A. *Tectonophysics* **47**, 43–58 (1978).
- Fujita, K. & Kanamori, H. *Geophys. J. R. astr. Soc.* **66**, 131–156 (1981).
- Kawakatsu, H. *Nature* **316**, 53–55 (1985).
- Abers, G. A. *Geophys. Res. Lett.* **19**, 2019–2022 (1992).
- Jordan, T. H. & Sverdrup, K. A. *Bull. Seism. Soc. Am.* **71**, 1105–1130 (1981).
- Kirby, S. H. *J. geophys. Res.* **92**, 13789–13800 (1987).
- Kirby, S. H., Durham, W. B. & Stern, L. A. *Science* **252**, 216–225 (1991).
- Green, H. W. & Burnley, P. C. *Nature* **341**, 733–737 (1989).
- Green, H. W., Young, T. E., Walker, D. & Scholz, C. H. *Nature* **348**, 720–722 (1990).
- Fuchs, K. & Muller, G. *Geophys. J. R. Astr. Soc.* **23**, 417–433 (1971).
- Kennett, B. L. N. *Seismic Wave Propagation in Stratified Media* 1–339 (Cambridge Univ. Press, 1983).
- Kawakatsu, H. *J. geophys. Res.* **91**, 6432–6440 (1986).
- Giardini, D. & Woodhouse, J. H. *Nature* **307**, 505–509 (1984).
- Yoshii, T. *Tectonophysics* **55**, 349–360 (1979).
- Sleep, N. H. *J. geophys. Res.* **84**, 4565–4571 (1979).
- Kawakatsu, H. *J. geophys. Res.* **91**, 4811–4825 (1986).
- Schubert, G., Yuen, D. A. & Turcotte, D. L. *Geophys. J. R. Astr. Soc.* **42**, 705–735 (1975).
- Vidale, J. E. & Benz, H. M. *Nature* **356**, 678–683 (1992).
- Sung, C. & Burns, R. G. *Tectonophysics* **31**, 1–32 (1976).
- Rubie, D. C. & Ross, C. R. *EOS* **73**, 378 (1992).
- Frohlich, C. A. *Rev. Earth planet. Sci.* **17**, 227–255 (1991).
- Helfrich, G. & Brodholt, J. *Nature* **352**, 252–255 (1991).
- Houston, H. & Williams, Q. *Nature* **352**, 520–522 (1991).
- Toksoz, M. N., Sleep, N. H. & Smith, A. T. *Geophys. J. R. Astr. Soc.* **35**, 285–310 (1973).
- Bina, C. R. & Wood, B. J. *J. geophys. Res.* **92**, 4853–4866 (1987).
- Akao, M., Ito, E. & Navrotsky, A. *J. geophys. Res.* **94**, 15671–15685 (1989).
- Goto, K., Suzuki, Z. & Hamaguchi, H. *J. geophys. Res.* **92**, 13811–13820 (1987).
- Bevis, M. *et al.* *EOS* **72**, 115 (1991).
- Richter, F. M. *J. geophys. Res.* **84**, 6783–6795 (1979).
- Dziewonski, A. M., Chou, T. & Woodhouse, J. H. *J. geophys. Res.* **86**, 2825–2852 (1981).

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Primary production control of methane emission from wetlands

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WETLANDS, both natural and agricultural, contribute an estimated 40 to 50% of the total methane emitted to the atmosphere each year. Recent efforts in atmospheric modelling¹ and attempts to constrain CH₄ source strengths² have indicated the need to delineate the processes responsible for the large variations in emission rates found within and across wetland types. Numerous biogeochemical factors are known to affect the activity of methanogenic bacteria^{3,4} and although there has been some success in relating water level⁵⁻⁷ and temperature^{8,9} to CH₄ emissions within particular systems, these variables are insufficient for predicting emissions across a variety of wetlands^{2,10}. From simultaneous measurements of CO₂ and CH₄ exchange in wetlands extending from subarctic peatlands to subtropical marshes, we report here a positive correlation between CH₄ emission and net ecosystem production and suggest that net ecosystem production is a master variable, integrating many factors which control CH₄ emission in vegetated wetlands. We find that about 3 per cent of the daily net ecosystem production is emitted back to the atmosphere as CH₄. With projected stimulation of primary production and soil microbial activity in wetlands associated with elevated atmospheric CO₂ concentrations¹¹, we envisage the potential for increasing CH₄ emissions from inundated wetlands, further enhancing the greenhouse effect.

Because many environmental factors are not entirely independent, it has been proposed¹⁰ that CH₄ emissions would best be correlated with variables that integrate environmental factors significant to CH₄ production, consumption, and emission. Evidence gathered in a subtropical marsh¹² and a northern boreal peatland¹³ has led us to consider net ecosystem production (NEP) as a variable meeting such requirements in water-saturated wetlands. To test the potential applicability of the CH₄ emission/NEP relationship across diverse wetland communities, we made measurements of CH₄ emission, net CO₂ exchange, and live above-ground biomass in nine wetland sites: five located in fens and bogs of Canada and Minnesota, with the remainder in temperate swamps, subtropical marshes and an agricultural rice field. Two Canadian peatland regions were studied during July and August 1990: an open fen covered predominantly with *Carex limosa* and *C. rostrata* (number of measurement plots, $n=10$), located near Schefferville, Quebec, Canada (55° N, 67° W)¹³, and in the Hudson Bay Lowlands, three wetlands, a coastal fen (51° N, 80° W; $n=4$) dominated by sedges and mosses and edged with tamarack, an interior fen (51° N, 81° W; $n=4$) also vegetated by sedges and mosses, and a plateau bog (51° N, 82° W; $n=6$) characterized by *Sphagnum* moss hummocks vegetated with ericaceous and coniferous shrubs and interspersed with wet sedge depressions. Bog and fen peatlands ($n=5$) were sampled near Red Lake, Minnesota (48° N, 95° W) during July 1991, with vegetation cover of *Sphagnum* and *Carex* sp. In the temperate and subtropical regions, we sampled *Typha latifolia* (cattail, $n=4$) in Virginia (37° N, 77° W) during June 1991 and also Florida Everglades *Typha* (*T. domingensis*, $n=7$; 25° N, 81° W) during January 1991. The predominant vegetation in the subtropical Everglades, *Cladium jamaicense* (sawgrass, $n=14$) was measured in October 1989 (ref. 12). Agricultural rice

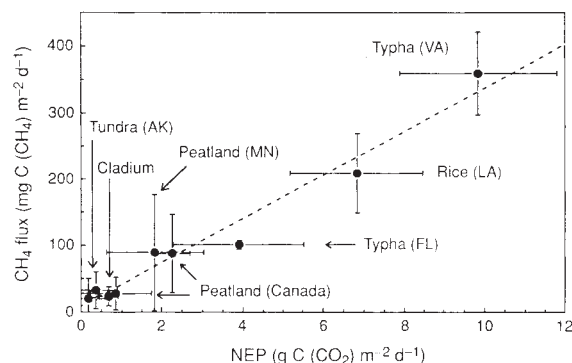


FIG. 1 Methane emission as a function of net ecosystem production (NEP). We simultaneously measured both CO₂ exchange and CH₄ emission with a closed, temperature-controlled, instrumented chamber¹⁴. This configuration measured the net exchange of CO₂ and CH₄ emission for the total system consisting of the above-ground foliage and the sediment/water surface. Changes in CO₂ concentrations within the chamber were measured by a portable infrared gas analyser (LICOR 6200). A minimum of six syringe samples were taken from the chamber's headspace over a 30-min period and analysed for methane concentrations within 1 to 2 h of collection on a gas chromatograph equipped with a flame ionization detector¹³. No tailing or flattening in the rate of methane emission was observed during these deployments⁴¹. We calculated NEP, which is equivalent to net primary production minus soil and microbial respiration, by applying a nonlinear model to concurrent measurements of net CO₂ exchange and photosynthetically active radiation¹⁴. The Alaskan tundra (AK) site (○) is shown for comparative purposes and was excluded from regression analysis. At each site, a mean was calculated from all plots for both CH₄ emission and CO₂ exchange and is expressed as amount per ground surface area. Error bars represent 95% confidence interval about the mean. The line is least-squares regression of mean values, slope is 0.033 g methane carbon/g CO₂ carbon, $r=0.98$, $n=9$.

(*Oryza sativa*, $n=12$) was sampled at the Rice Research Station of Louisiana State University (30° N, 92° W) during the grain-ripening stage in July 1991. With the exception of the bog sites, which had surfaces 20 to 50 cm above the water table, water levels ranged from 5 to 20 cm above the sediment. For comparison purposes, we have also included an Alaskan tundra site (Fig. 1; Tundra, AK) calculated from a combination of chamber^{14,15} and micrometeorological tower¹⁶ measurements.

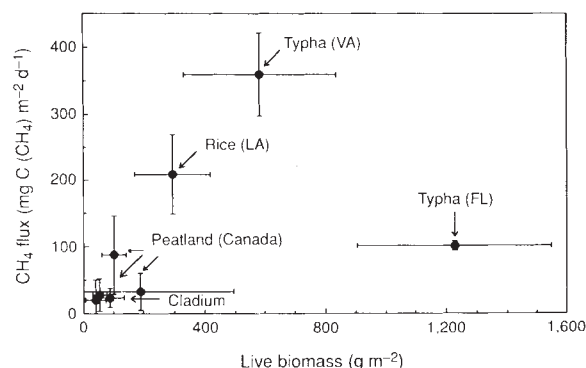
When methane emissions are plotted against NEP, the result is a linear relationship across wetland types (Fig. 1). Variability of emissions among both natural and agricultural sites is strongly correlated to differences in primary production. This relationship also holds for low emissions in tundra regions, in addition to CH₄ emissions from Minnesota peatlands which are considered to be anomalously high⁸ compared with values obtained in similar Canadian wetlands^{6,7}.

The relationship between CH₄ emission and NEP may be related to the stimulation of methanogenesis by the increase of substrates associated with recent production, including root exudation^{9,17,18} and turnover¹⁹, and litter input^{20,21}. Variations in below-ground methane concentration correlated with plant presence in peatlands¹³ and the enhancement of CH₄ production and emission associated with rice plants^{18,22} indicate the importance of below-ground production. Results of laboratory experiments²³ suggest the potential of exudation products²⁴ to support methanogenesis.

The relationship observed (Fig. 1) may also be related to the action of plants providing conduits for gas exchange. In the process of aerating below-ground organs, plants ventilate methane²⁵. The apparent correlation between CH₄ emission and live above-ground biomass (Fig. 2) for most of these sites (notable exception: *Typha*, FL) is consistent with this conduit effect. Generally, methane is released from plant stems or culms rather

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FIG. 2 Methane emission as a function of live above-ground biomass removed after CH_4 measurements from specific sites as in Fig. 1 and dried (60°C).



than from leaves^{17,26,27}; therefore, leaf stomatal activity (that is, concomitant CO_2 uptake and CH_4 release through stomates) is not a major factor affecting the observed relationship between CO_2 exchange and CH_4 release^{17,28}.

Biomass, besides acting as a measurement surrogate for conduit potential (related to stem cross-sectional area and root biomass²⁹⁻³¹, parameters important in gas transport^{25,26}), is also related to the ecosystem's potential to fix carbon. It is noteworthy that methane emission for the apparent outlying community (*Typha*, FL; Fig. 2) with high conduit potential (high biomass) converges with the other sites when related to NEP (Fig. 1), suggesting that NEP is a stronger controlling variable than biomass. The strength of primary production as a predictor of CH_4 emission was also demonstrated by its ability to unite two disparate emission versus biomass data sets in a wetland sedge¹², its relation to emission across successional peatland gradients³², and its success in modelling atmospheric distributions of methane¹.

Previous efforts to constrain the range of CH_4 emissions from wetlands by using the ratio of emissions to net primary production (equal to NEP plus soil microbial respiration) have been hindered by the lack of concurrent net primary production and methane emission measurements². Using available data, it has been estimated² that the ratio of CH_4 emission to net primary production should be well below 5%. The relationship between emission and carbon input derived in this study indicates that 3% of the NEP during peak production periods was emitted back to the atmosphere as CH_4 . It is possible that this ratio may vary with seasonal changes in net carbon exchange, although seasonal measurements (G.J.W. and J.P.C., unpublished results) in a temperate *Typha* marsh suggest consistency.

The failure of temperature to predict consistently CH_4 emissions in the field may be related to changes in substrate availability. The direct response of methanogenesis to temperature changes has been shown in laboratory studies to have Q_{10} values ranging from 2.5 to 3.5 (refs 4, 33, 34). The extent to which methanogenesis in the field is directly regulated by temperature is less defined. In some field measurements, emissions were not correlated with temperature^{35,36}, or correlations were dependent upon plant community type^{7,37,38}. Often, correlations of seasonal emission and temperature measurements from the field have resulted in Q_{10} values ranging between 4 and 13 (refs 8, 9, 34, 38), well above laboratory-derived values. Both field and laboratory results^{33,34} indicate that increased organic carbon input can elevate apparent Q_{10} values. We propose that the evidently high Q_{10} values derived from seasonal field measurements may be the result of seasonal changes in photosynthetic production and substrate availability for methanogenesis in addition to temperature effects.

The relationship derived between primary production and methane emission provides an important tool for refining global scale source estimates. Satellite-derived remote sensing indices have been related to photosynthetic activity³⁹ and net primary production over large regions⁴⁰. The use of these remote sensing

relationships as inputs for biogeochemical models of trace gas emissions will necessitate a more complete understanding of how mechanisms that regulate emission (such as substrate quality and production, water level and temperature) integrate within and vary across wetlands, but we are encouraged by these initial results (Fig. 1) which suggest the feasibility of developing such capabilities. □

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1. Taylor, J. A., Brasseur, G. P., Zimmerman, P. R. & Cicerone, R. J. *J. geophys. Res.* **96**, 3013-3044 (1991).
2. Aselmann, I. & Crutzen, P. J. *J. atmos. Chem.* **8**, 307-358 (1989).
3. Rudd, J. W. M. & Taylor, C. D. *Adv. aquat. Microbiol.* **2**, 77-150 (1980).
4. Conrad, R. in *Exchange of Trace Gases between Terrestrial Ecosystems and the Atmosphere* (eds Andreae, M. O. & Schimel, D. S.) 39-58 (Wiley, New York, 1989).
5. Harriss, R. C., Sebachner, D. I. & Day, F. P. *Nature* **297**, 673-674 (1982).
6. Moore, T., Roulet, N. & Knowles, R. *Global biogeochem. Cycles* **4**, 29-46 (1990).
7. Roulet, N. T., Ash, R. & Moore, T. R. *J. geophys. Res.* **97**, 3739-3749 (1992).
8. Crill, P. M. et al. *Global biogeochem. Cycles* **2**, 371-384 (1988).
9. Schutz, H., Holzapfel-Pschorn, A., Conrad, R., Rennenberg, H. & Seiler, W. *J. geophys. Res.* **94**, 16405-16416 (1989).
10. Whalen, S. C. & Reeburgh, W. S. *Global biogeochem. Cycles* **6**, 139-160 (1992).
11. Lamborg, M. R., Hardy, R. W. F. & Paul, E. A. in *CO₂ and Plants: The Response of Plants to Rising Levels of Atmospheric Carbon Dioxide* (ed. Lemon, E. R.) 131-176 (AAAS Selected Symp. 84, 1983).
12. Whiting, G. J., Chanton, J., Bartlett, D. & Happell, J. *J. geophys. Res.* **96**, 13067-13071 (1991).
13. Whiting, G. J. & Chanton, J. P. *Global biogeochem. Cycles* **6**, 225-231 (1992).
14. Whiting, G. J., Bartlett, D. S., Fan, S. M., Bakwin, P. & Wofsy, S. J. *geophys. Res.* **97**, 16671-16680 (1992).
15. Bartlett, K. B., Crill, P. M., Sass, R. L., Harriss, R. C. & Dise, N. B. *J. geophys. Res.* **97**, 16645-16660 (1992).
16. Fan, S. M. et al. *J. geophys. Res.* **97**, 16627-16643 (1992).
17. Seiler, W., Holzapfel-Pschorn, A., Conrad, R. & Scharffe, D. *J. atmos. Chem.* **1**, 241-268 (1984).
18. Holzapfel-Pschorn, A., Conrad, R. & Seiler, W. *Plant and Soil* **92**, 223-233 (1986).
19. Van Veen, J. A., Merckx, R. & Van de Geijn, S. C. *Plant and Soil* **115**, 179-188 (1989).
20. Cicerone, R. J., Delwiche, C. C., Tyler, S. C. & Zimmerman, P. R. *Global biogeochem. Cycles* **6**, 233-248 (1992).
21. Valentine, D. W., Holland, E. A. & Schimel, D. S. *J. geophys. Res.* (in the press).
22. Sass, R. L., Fisher, F. M. & Harcombe, P. A. *Global biogeochem. Cycles* **4**, 47-68 (1990).
23. Jeris, J. S. & McCarthy, P. L. *J. water poll. contr. Fed.* **37**, 178-192 (1965).
24. Hale, M. G., Moore, L. D. & Griffin, G. J. in *Interactions Between Non-pathogenic Soil Microorganisms and Plants* (eds Dommergues, Y. R. & Krupa, S. V.) 163-203 (Elsevier, New York, 1978).
25. Chanton, J. P. & Dacey, J. W. H. in *Trace Gas Emissions from Plants* (eds Sharkey, T., Holland, E. & Mooney, H.) 65-92 (Academic, San Diego, 1991).
26. Schutz, H., Schroder, P. & Rennenberg, H. in *Trace Gas Emissions from Plants* (eds Sharkey, T., Holland, E. & Mooney, H.) 29-64 (Academic, San Diego, 1991).
27. Nouchi, I., Mariko, S. & Aoki, K. *Plant Physiol.* **94**, 59-66 (1990).
28. Chanton, J. P., Whiting, G. J., Showers, W. J. & Crill, P. M. *Global biogeochem. Cycles* **6**, 15-31 (1992).
29. Whigham, D. F. & Simpson, R. L. *Aquat. Bot.* **5**, 355-364 (1978).
30. Gross, M. F., Hardisky, M. A., Wolf, P. L. & Klemas, V. *Estuaries* **14**, 180-191 (1991).
31. Arenovski, A. L. & Howes, B. L. *Oecologia* **90**, 316-322 (1992).
32. Klinger, L. F., Zimmerman, P. R., Greenberg, J. P., Heidt, L. E. & Guenther, A. B. *J. geophys. Res.* (in the press).
33. Conrad, R., Schutz, H. & Babbel, M. *FEMS microbiol. Ecol.* **45**, 281-289 (1987).
34. Kelly, C. A. & Chynoweth, D. P. *Limnol. Oceanogr.* **26**, 891-897 (1981).
35. Yavitt, J. B., Lang, G. E. & Sextone, A. J. *J. geophys. Res.* **95**, 22463-22474 (1990).
36. Svensson, B. H. *Ecol. Bull.* **30**, 235-250 (1980).
37. Svensson, B. H. & Rosswall, T. *Ecol. Bull.* **30**, 283-301 (1980).
38. Morrissey, L. A. & Livingston, G. P. *J. geophys. Res.* **97**, 16661-16670 (1992).
39. Cihlar, J., Caramori, P. H., Schuepp, P. H., Desjardins, R. L. & MacPherson, J. I. *J. geophys. Res.* **97**, 18515-18521 (1992).
40. Goward, S. N., Tucker, C. J. & Dye, D. G. *Vegetatio* **64**, 3-14 (1985).
41. Chanton, J. P., Whiting, G. J., Happell, J. D. & Gerard, G. *Aquat. Bot.* (in the press).

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Disparity-sensitive cells in the owl have a characteristic disparity

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WE experience the visual world as being three-dimensional. A major source of depth information derives from the slightly different views of each eye, leading to small variations in the retinal images ('disparities'). Neurons sensitive to visual disparities are thought to form the neural basis of stereo vision^{1,10}. Barn owls^{2,3} as well as several mammalian species^{1,4-10} have neurons that are sensitive to visual disparities. But how visual disparities are represented in the brain has been a matter of discussion ever since the first disparity-sensitive neurons were found some 25 years ago. Here we adopt a new approach to this problem and study the neural computation of visual disparities with a paradigm borrowed from auditory research. The measurement of interaural time difference (ITD) has many similarities with the measurement of visual disparity on the formal, algorithmic level. We speculate that the similarities might extend to the level of neural computation. The neural representation of ITD is well understood¹¹⁻¹⁸, and we have studied the representation of disparities with visual stimuli analogous to those successfully used in acoustic experiments. For example, ITD is converted in the brain to a pathlength on an axon that, owing to the finite conduction velocity in neurons, exactly matches the external ITD. This pathlength is called 'characteristic delay'¹². Our results suggest that there is an analogue of the characteristic delay in stereo vision which we propose to call 'characteristic disparity'.

We were interested in the mechanisms underlying the measurement of disparities in the barn owl (*Tyto alba*). Our experimental approach was to make use of the similarities—on the formal, algorithmic level—in the measurement of interaural time difference, a cue signalling sound-source azimuth, and of visual disparities. In both cases the interaction of signals coming from two spatially separated sensors is necessary. A small shift of the important variable, position or time, has to be detected. A correspondence problem exists for both tasks; that is, correct matches of the events in the two signals have to be discriminated from incorrect matches. The underlying algorithms are well understood at the psychophysical level¹⁹⁻²¹, but more is known about the neural computation of ITD than of visual disparities.

ITD is detected in neurons that function as coincidence detectors. The path from the ear to a coincidence-detecting neuron is slightly longer on the side that hears the stimulus first^{11,12,15,18}. Owing to the finite conduction velocity in neurons, the difference in pathlength is converted to an ITD. The time consumed by the longer path exactly corresponds to the ITD of the signal^{15,18}. This difference in time (or path length) has been termed 'characteristic delay'¹². As the characteristic delay is determined by a pathlength, it is independent of stimulus frequency. The characteristic delay can be measured in two ways: first, if a neuron is sufficiently broadly tuned to frequency, and noise is used as a stimulus, the response as a function of ITD, the ITD curve, shows one dominant peak (Fig. 1a; see also refs 14, 16). This 'main' peak is accompanied by side-peaks, the height of which may vary from being almost absent (Fig. 1a) to nearly reaching the height of the main peak¹⁴. In most ITD-sensitive neurons of the owl, the characteristic delay occurs at the main peak^{14,16}. By contrast, if a tone is used as stimulus, the ITD curve has several maxima, but for different frequencies maxima are only coincident at the characteristic delay (Fig. 1b; see also refs 14, 16-18).

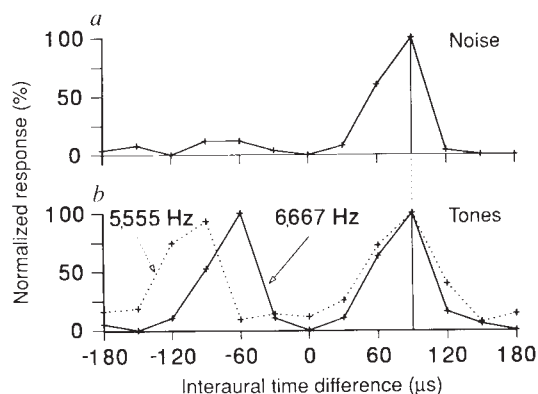


FIG. 1 Characteristic delay in an ITD-sensitive auditory cell. *a*, The response to stimulation with broad-band noise (0–25 kHz) showed one main peak and provided only a slight suggestion of the position of the side peaks that were expected to occur around -30 to -90 μ s. *b*, The responses to stimulation with tones exhibited cyclicity. The relative response amplitude at the peak occurring at 90 μ s was independent of frequency. This peak corresponded to the characteristic delay^{12,14}. Stimuli were bursts of 100 ms having a rise/fall time of 5 ms. Interaural time differences are negative when the sound to the left ear is leading but positive when the sound to the right ear is leading. One degree in auditory space corresponds to 2–2.5 μ s. For more information on auditory stimulation and data analysis, see ref. 16. Note that this is not a coincidence-detecting cell, but that it was located further up in the auditory pathway, precisely in the external nucleus of the inferior colliculus. The characteristic delay can be seen anyway, because it is conserved in the ascending auditory pathway. The maxima of the response curves (per 5 stimulus repetitions) were 25 spikes (noise), 19 spikes (6,667 Hz), and 21.5 spikes (5,555 Hz).

In our experiments we wanted to find out whether disparity-sensitive cells have an analogue of the characteristic delay which we shall call 'characteristic disparity'.

Disparity-tuning curves, or responses of a cell as a function of disparity, were recorded at 58 sites in the avian analogue of the visual cortex, the visual Wulst^{2,22}, of three barn owls. The preferred orientations of the neurons ranged from -60 to $+60$ degrees relative to vertical. All experiments were done under anaesthesia as described earlier¹⁶. The head of the owl was fixed to the set-up via a head-holder cemented onto the skull. Animals were not paralysed as eye movements are negligible in owls^{2,3,23}. Stimuli were constructed to be analogous to the auditory stimuli previously used to study the neural representation of ITD. The visual stimuli were either sinewave gratings (46 tuning curves) or patterns having a randomly varying luminance in the optimal motion direction ('one-dimensional visual noise') (25 tuning curves).

The spatial-frequency tuning in the Wulst was similar to that in the mammalian visual cortex^{6,7,20,24} and ranged from one to more than three octaves. In response to binocular stimulation with noise, disparity-tuning curves showed several response peaks, but in most cases one peak was more pronounced than the others (Fig. 2a). This 'main' peak was flanked by side-peaks (Fig. 2a). In response to binocular stimulation with sinewave gratings at various disparities, disparity-tuning curves showed a cyclic response. In our stimulus paradigm, one cycle of the response was physically linked to one cycle of the stimulus, because cells could not discriminate shifts of the sinewave gratings of exactly one period. Thus, when spatial frequency was changed, the spacing of the peaks changed as well. Note that not all of the peaks shifted alike as spatial frequency was changed. For this neuron, one peak was always found around a disparity of $+2$ degrees, independently of spatial frequency. In contrast, the response peak occurring to the left changed its position with spatial frequency. Thus, by analogy with the audi-

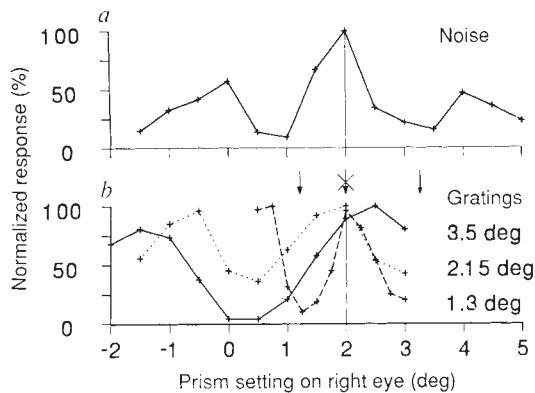


FIG. 2 Characteristic disparity in a disparity-sensitive visual cell. *a*, Stimulation with visual noise; *b*, stimulation with gratings having a spatial wavelength of 3.5°, 2.15° and 1.3°. As the patterns were inclined 30° to the vertical and disparity was only changed in the horizontal direction, the effective periods were 4, 2.5 and 1.5°, respectively, and these were also the periods corresponding to one cycle of the responses. Note that one peak is prominent in *a*. The response at this disparity was always close to maximal if gratings were used as a stimulus (*b*). Thus, the CD was close to the main peak. Negative values of the prism settings correspond to displacements towards uncrossed disparities (see Methods). The cross indicates the expected position of the peaks when the CD model is fulfilled; arrows indicate the expected positions when the phase model is fulfilled (see Fig. 3 legend). The maximum response was 299 spikes (noise), 223 spikes (1.3°), 617 spikes (2.15°), and 191 spikes (3.5°).

METHODS. Visual stimuli were created on a Silicon Graphics IRIS 4D/310GTX computer and projected onto a translucent screen located 85 cm in front of the owl by an Electrochrome ECP4000 high-resolution colour projection system. Stimuli were presented in a 10°-wide circular aperture. They were aligned in the optimal orientation and moved in the preferred direction at the optimal velocity. About 3 cm in front of the owl's eyes (pupillary separation 40 mm) was mounted a trial lens spectacle frame. Disparity was changed in a pseudorandom fashion with the help of a Risley prism placed in the spectacle frame in front of the right eye. To correct for the distance of the stimuli, lenses of 1 dpt were mounted in the spectacle frame in front of each eye. Before starting the neurophysiological recordings, retinal landmarks (the pecten oculi and the ora terminalis) were backprojected onto the translucent screen, and their position on the screen was recorded. Because the foveae are not visible ophthalmoscopically in barn owls, we could not determine the visual axis during an experiment, but earlier data^{2,3} suggested that these landmarks provide a useful frame of reference to monitor eye movements. Thus, our prism settings do not reflect absolute values of disparity. Note that for our conclusions it is only important that eye movements are excluded. This condition is fulfilled in that (1) the owl's eyes are virtually immobile in the skull²³, and (2) we and others^{2,3} did not observe eye movements when replotting the position of the tip of the pecten oculi several times during an experiment.

tory data, these changes in the tuning curves suggested that the cell shown in Fig. 2 indeed had a characteristic disparity (CD) of about +2°. This conclusion was supported by the second criterion mentioned above, because the main peak in the disparity curves obtained with noise stimulation also occurred at a disparity of +2° (Fig. 2*a*). When two excitatory signals interact, the characteristic delay of auditory neurons occurs at the peak of the ITD curve, but when one excitatory and one inhibitory signal interact, the characteristic delay is found at the trough^{12,14,16}. If the CD occurred at the trough, the response at the trough should be frequency-independent, and the position of both the main peak and the neighbouring peaks should shift by similar amounts, whereas if the CD is found at a peak, this peak should scatter around a mean disparity when frequency is changed, and only the neighbouring peaks should shift. Because in our sample of disparity-sensitive cells the position of the main peak was frequency independent, while the neighbouring peaks

shifted (Fig. 3*a*), the CD occurred at the main peak.

The CDs we encountered ranged from 0–2.5° of disparity and could span 2° in one recording session. This range by far exceeded half of the effective spatial wavelengths, which could be as low as 0.65° (Fig. 2*b*). In some visual experiments (16 cases), a moving bar, the classical stimulus for determining disparity tuning, was used as stimulus. In many of these cases the disparity-tuning curves exhibited one dominant response peak, and this peak was also close to or at the CD.

The results we have presented so far may still be consistent with a variety of models of the neural implementation of disparity. In disparity-sensitive neurons, the response of at least two inputs have to interact. The receptive fields (RFs) of the input cells can be modelled by Gabor filters, and different shapes of the RFs arise by phase shifts. Further analysis was carried out to discriminate between two specific models. One of these models (the 'phase' model) is based on the interaction of monocular receptive fields having different shapes but equal centres²⁵; the second is our proposal that cells have a CD at the main peak

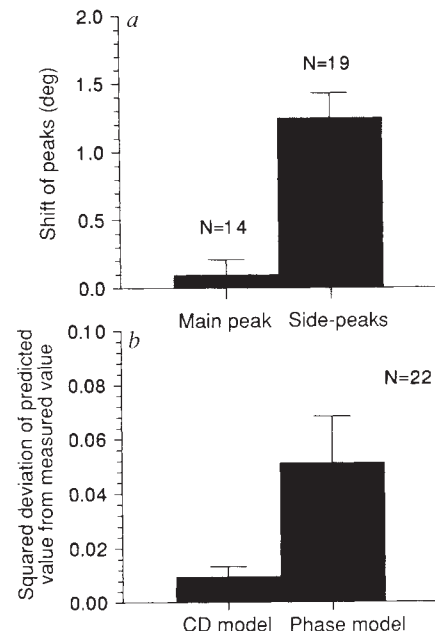


FIG. 3 Assessment of data variability. For the analyses shown, the disparity-tuning curves were fitted by a cosine function with a nonlinear fitting procedure. Mean values and standard deviations of the means are plotted. *a*, The position of the main peak is much more stable than the position of the side-peaks, suggesting that the characteristic disparity occurred at or near the main peak. *b*, Discrimination between the phase model and the CD model. For this analysis, first the mean value of the disparity at the main peak was computed by using all data recorded with noise and spatial frequencies to provide a better estimate of the disparity to which a cell was tuned than in the individual tuning curves. Then, the best frequency, namely the frequency that elicited the highest response, was used to calculate the phase value belonging to the mean disparity, the mean binocular phase difference (MP). With the mean disparity (MD), MP, the actually measured disparity and the effective periods (EP; see also legend to Fig. 2), we could make predictions concerning the two different models: (1) the quotient MD/EP reflects the predicted binocular phase difference for the CD model; and (2) MP is the prediction for the phase model. The squared deviations between the actually measured binocular phase differences and the predicted binocular phase difference served as an indicator for the goodness of the models. The analysis shows that deviations from the predictions are much higher under the assumption that the phase model is fulfilled (*b*). Note that the models cannot be discriminated at the stimulation frequency that is used as a reference (here, the best frequency). Thus the cross and one arrow coincide in Fig. 2*b*.

(the 'CD' model), which would suggest that the interacting cells have equal RF shapes but shifted centres. For cells tuned to disparities differing from zero, the two models can, in principle, be discriminated, because the phase model predicts a dependence of the main peak on spatial frequency, whereas the CD model predicts frequency independence. The analysis of our sample strongly favours the CD model over the phase model, because the squared deviations of the predicted values from the measured values (for details see legend of Fig. 3) are much larger for the phase model than for the CD model (Fig. 3b) (t -test, $n=22$, $t=2.55$, $P<0.02$). An even more striking result was found if the differences in the individual cases were compared (Wilcoxon matched pairs signed rank test; $n=22$, smaller sum: 30, $P<0.001$). Because of noise in the data, the two models can, in practice, not be distinguished for characteristic disparities close to zero. The phase model was favoured in five cases, and, indeed, three of these five cases were found when the main peak had a disparity of less than 0.05 degrees.

These experiments reveal that disparity-sensitive cells in the owl's Wulst have a CD. The CD was detected by stimulating each cell with a wide variety of spatial frequencies. As the stereo mechanism in the owl is in many respects similar to that in mammals²⁶, it may be speculated that disparity-sensitive cells in the visual cortex also have a CD. Unfortunately, adequate experiments have not been done in any mammal so far. But our model is consistent with earlier data suggesting that disparities can be represented as a shift in retinal position of equally shaped monocular RFs of a binocular cell^{5,27}. Limitations of our approach are that it might be difficult to test cells with a preferred orientation close to horizontal. Our sample did not include neurons tuned to horizontal orientations. Thus we cannot say whether such neurons also have a characteristic disparity.

We started with two problems that are formally similar and used the better understood neural implementation of one to study the neural implementation of the other. We suggest that such a comparison between sensory modalities may be fruitful in other systems as well. Specifically we want to test whether the neural representation of these two algorithms does have further similarities, for example in the solution of the correspondence problem. □

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- Barlow, H. B., Blakemore, C. & Pettigrew, J. D. *J. Physiol.* **193**, 327–342 (1967).
- Pettigrew, J. D. & Konishi, M. *Science* **193**, 675–678 (1976).
- Pettigrew, J. D. *Proc. R. Soc. Lond.* **B204**, 435–454 (1979).
- Maske, R., Yamane, S. & Bishop, P. O. *Vision Res.* **24**, 1921–1929 (1984).
- Poggio, G. F., Motter, B. C., Squatrito, S. & Trotter, Y. *Vision Res.* **25**, 397–406 (1985).
- Ohzawa, I. & Freeman, R. D. *J. Neurophysiol.* **56**, 221–242 (1986).
- Ohzawa, I. & Freeman, R. D. *J. Neurophysiol.* **56**, 243–259 (1986).
- Ohzawa, I., deAngelis, G. C. & Freeman, R. D. *Science* **249**, 1037–1041 (1990).
- deAngelis, G. C., Ohzawa, I. & Freeman, R. D. *Nature* **352**, 156–159 (1991).
- Hammond, P. *Expl Brain Res.* **87**, 615–623 (1991).
- Jeffress, L. A. *J. comp. Phys. Psychol.* **41**, 35–39 (1948).
- Rose, J. E., Grass, N. G., Geisler, C. D. & Hind, J. E. *J. Neurophysiol.* **29**, 288–314 (1966).
- Yin, T. C. T. & Kuwada, S. in *Dynamic Aspects of Neocortical Function* (eds Edelman, G. M., Gall, W. E. & Cowan, W. M.) 263–313 (Wiley, New York, 1984).
- Takahashi, T. & Konishi, M. *J. Neurosci.* **6**, 3413–3422 (1986).
- Sullivan, W. E. & Konishi, M. *Proc. natn. Acad. Sci. U.S.A.* **83**, 8400–8404 (1986).
- Wagner, H., Takahashi, T. & Konishi, M. *J. Neurosci.* **7**, 3105–3116 (1987).
- Konishi, M., Takahashi, T., Wagner, H., Sullivan, W. E. & Carr, C. E. in *Auditory Function* (eds Edelman, G. M., Gall, W. E. & Cowan, W. M.) 721–745 (Wiley, New York, 1988).
- Carr, C. E. & Konishi, M. *J. Neurosci.* **10**, 3227–3246 (1990).
- Julesz, B. *Foundations of Cyclopean Perception* (University of Chicago Press, Illinois, 1971).
- Blake, R. & Wilson, H. R. *Trends Neurosci.* **14**, 445–452 (1991).
- Moiseff, A. J. *comp. Physiol.* **A 164**, 637 (1989).
- Karten, H. J., Hodos, W., Nauta, W. J. H. & Revzin, A. M. *J. comp. Neurol.* **150**, 253–278 (1973).
- Steinbach, M. J. & Money, K. E. *Vision Res.* **13**, 889–891 (1973).
- Movshon, J. A., Thompson, I. D. & Tolhurst, D. J. *J. Physiol.* **263**, 101–120 (1978).
- Nomura, M., Matsumoto, G. & Fujiwara, S. *Biol. Cybern.* **63**, 237–242 (1990).
- Pettigrew, J. D. in *Vision: Coding and Efficiency* (ed. Blakemore, C.) 283–290 (Cambridge University Press, UK, 1990).
- Hubel, D. H. & Wiesel, T. N. *J. Physiol.* **160**, 106–154 (1962).

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Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by *Listeria monocytogenes*

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TUMOUR necrosis factor (TNF), jointly referring to TNF α and TNF β , is a central mediator of immune and inflammatory responses; its activities are mediated by two distinct receptors, TNFR1 (p55) and TNFR2 (p75) (reviewed in refs 1–3). The cytoplasmic domains of the TNFRs are unrelated, suggesting that they link to different intracellular signalling pathways⁴. Although most TNF responses have been assigned to one or the other of the TNF receptors (mostly TNFR1), there is no generally accepted model for the physiological role of the two receptor types. To investigate the role of TNFR1 in beneficial and detrimental activities of TNF, we generated TNFR1-deficient mice by gene targeting. We report here that mice homozygous for a disrupted *Tnfr1* allele (*Tnfr1*⁰) are resistant to the lethal effect of low doses of lipopolysaccharide after sensitization with D-galactosamine, but remain sensitive to high doses of lipopolysaccharide. The increased susceptibility of *Tnfr1*⁰/*Tnfr1*⁰ mutant mice to infection with the facultative intracellular bacterium *Listeria monocytogenes* indicates an essential role of TNF in nonspecific immunity.

The gene targeting vector consisted of a genomic mouse DNA fragment⁵, in which exons 2, 3 and part of exon 4 of the *Tnfr1* gene were replaced by a *neo*^r cassette (Fig. 1a–c). This deletion disrupts the gene and removes the coding information for the cysteine-rich domains I and II of the receptor which have been shown to be essential for ligand binding⁶. Germ-line transmitters of the mutated *Tnfr1* allele were crossed with C57BL/6 mice and the resulting heterozygous mice interbred to yield homozygous mutant offspring. The F₁ generation displayed the expected mendelian 1:2:1 ratio of wild-type (+/+), heterozygous (0/+) and homozygous (0/0) mutant mice. This indicates that *Tnfr1* expression is not required for normal embryonic development. B6x129-*Tnfr1*⁰/*Tnfr1*⁰ mice are viable, breed normally and show no apparent phenotypic anomalies at the present state of investigation. The inactivation of the *Tnfr1* gene was confirmed by Southern blot analysis (Fig. 1d), and by northern blot analysis with RNA from different tissues of homozygous mutants which revealed the lack of *Tnfr1* message (Fig. 1e). Absence of expression of TNFR1 protein in the mutants was demonstrated by the lack of binding of iodinated human TNF α , specific for mouse TNFR1 (ref. 7) (Fig. 1f). Mutant mice, however, still express TNFR2 as revealed by binding mouse ¹²⁵I-TNF α .

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TABLE 1 Lethal toxicity of LPS and TNF α in *Tnfr1*⁰/*Tnfr1*⁰ mutants and wild-type control mice

(a) Mice treated with LPS plus D-GalN

Genotype	D-Galactosamine (mg)	LPS <i>Salmonella enteritidis</i> (μ g)	Lethality (deaths/total)
Wild-type	8	0.001	2/6
	8	0.010	9/9
	8	0.030	5/5
	8	0.100	5/5
<i>Tnfr1</i> ⁰ / <i>Tnfr1</i> ⁰	8	0.010	0/5
	8	0.100	0/5
	8	1.000	0/5

(b) Mice treated with LPS alone

Genotype	LPS <i>S. enteritidis</i> (μ g)		LPS <i>S. abortus equi</i> (μ g)	
		Lethality (deaths/total)		Lethality (deaths/total)
Wild-type	100	0/3	100	0/5
	300	0/3	300	3/10
	600	1/4	600	4/10
	1,200	5/7	1,000	7/8
<i>Tnfr1</i> ⁰ / <i>Tnfr1</i> ⁰	50	0/5	300	0/5
	1,200	5/5	600	2/5
			1,000	4/5

(c) Mice treated with recombinant mouse TNF α (rmTNF α) plus recombinant human IL1 α (rhIL1 α)

Genotype	TNF α plus IL1 α (μ g)	Lethality (deaths/total)
Wild-type	1.0 + 1.0	3/5
<i>Tnfr1</i> ⁰ / <i>Tnfr1</i> ⁰	1.0 + 1.0	0/5

LPS (*Salmonella enteritidis*, Sigma, Cat. no. L 6011 or *S. abortus equi*, SEBAK, Munich Germany) and D-GalN (Sigma, Cat. no. G 0264) were injected intraperitoneally into mice in 0.2 ml pyrogen-free 0.9% saline. LPS in mice without D-GalN sensitization and the mixture of rmTNF α (Hoffmann-La Roche, Basel, Switzerland) and of rhIL1 α (Hoffmann-La Roche, Nutley, USA) were administered intravenously. The dose of LPS was calculated per 20 g body weight. All mice (8–12 weeks of age) were kept under specific pathogen-free (spf) conditions. D-GalN-sensitized control mice died 6–9 h after LPS challenge, cytokine-treated control mice 7 h after challenge. Mortality of non-sensitized mice was recorded over 4 days after challenge. Sensitization of mice was repeated with 20 mg D-GalN per mouse with similar results.

The development of lymphocyte subpopulations in *Tnfr1*⁰/*Tnfr1*⁰ mutants was investigated by cytofluorometric analysis. There were normal numbers of both T and B cells in lymphatic organs. Using anti-CD4, CD8 and CD3 antibodies, no significant changes in relative size of major subpopulations could be detected (not shown). Thus, expression of TNFR1 does not appear to be essential for the normal development of T lymphocytes.

Sepsis and septic shock are systemic responses with high mortality to infection⁸. The endogenous mediators TNF α and interleukin-1 (IL-1) released in response to lipopolysaccharide (LPS) and other bacterial products have been identified as the principal mediators of the pathology in sepsis (reviewed in refs 1–3). It has been shown that D-galactosamine (D-GalN) dramatically sensitizes mice to the lethal effect of LPS⁹ due to its toxic effects on hepatocytes. There is agreement that death in LPS/D-GalN-challenged mice is due to TNF toxicity^{10–12}. In our studies with D-GalN-sensitized B6x129 wild-type mice, lethality could be induced with LPS (*Salmonella enteritidis*) doses of as little as 1 ng and reached 100% at 10 ng LPS (Table 1a). In contrast, D-GalN-sensitized *Tnfr1*⁰/*Tnfr1*⁰ mice were completely insensitive to the lethal effect of LPS, even when it was given at a dose more than 100 times greater than the lethal dose for wild-type littermates. To assess the involvement of TNFR1 in TNF systemic toxicity directly, we challenged the mice with TNF α in the presence of IL-1 α which was shown to potentiate the lethal effect of TNF α ¹³. Although this treatment severely affected all wild-

type mice and caused death in 3 out of 5 animals, *Tnfr1*⁰/*Tnfr1*⁰ mutant mice were without any symptoms of illness (Table 1c). These experiments demonstrate that TNF α signalling through TNFR1 is necessary for the lethal pathology induced indirectly by LPS in D-GalN-sensitized mice or directly with TNF α potentiated by IL-1 α . We cannot, however, formally exclude a role of TNFR2 in TNF systemic toxicity¹⁴ because TNFR2 involvement might depend on a TNFR1-mediated co-signal. We also challenged the mutant mice with sufficiently high doses of LPS to cause death without D-GalN sensitization. LPS doses needed for lethality in normal mice in the absence of D-GalN was found to be 300–1,200 μ g using LPS from two different sources, namely *Salmonella enteritidis* and *S. abortus equi* (Table 1b). These LPS doses, which are about 10⁵ higher than those given to D-GalN-sensitized mice, were also lethal for *Tnfr1*⁰/*Tnfr1*⁰ mutant mice. These results show that pathways other than TNFR1-dependent become operational in lethality induced in the LPS toxicity model.

Administration of LPS to mice induces macrophages to produce and secrete the pro-inflammatory cytokines TNF α , IL1 and IL6, which are released sequentially into the circulation (reviewed in ref. 15). We have followed the kinetics of systemic TNF α and IL-6 release in *Tnfr1*⁰/*Tnfr1*⁰ mutants and wild-type littermates after challenge with a sublethal dose of LPS. The peak concentration of TNF α was found to be slightly enhanced, if changed at all, in mutant as compared with control animals (Fig. 2a). Secretion of IL-6, however, was diminished about threefold in TNFR1-deficient mice (Fig. 2b), suggesting a cascade of events in which TNFR1-mediated signals are involved in triggering subsequent IL-6 secretion; to a lower extent, however, IL-6 was induced, with the same kinetics, through a TNFR1-independent pathway^{16,17}.

Early host defence against facultative intracellular pathogens is thought to be mediated by TNF, interferon- γ (IFN- γ) and other cytokines which are involved in the local control of the infection. Administration of TNF α neutralizing antibodies suppresses the ability of mice to control the intracellular bacterium *Listeria monocytogenes*^{18–20}. *Tnfr1*⁰/*Tnfr1*⁰ mutant mice therefore were infected intravenously with 4.0 $\times$ 10³ colony-forming units (CFU) of *L. monocytogenes* (a sublethal dose for wild-type mice). Four days after infection mutant mice exhibited 5,000–500,000-fold increased bacterial titres in spleen and liver when compared with control mice (Fig. 3a). On day 7 after infection all of four mutant mice, but none of the controls, have died. Even a challenge with only 250 CFU *L. monocytogenes* cannot be controlled by the mutant animals (Fig. 3a); by day 6 after infection again all mutant mice were dead. Hence, TNFR1-deficient mice are unable to clear bacteria from infected organs and eventually die from uncontrolled listeriosis. Together with findings in IFN γ R-deficient mice²¹, these results indicate that signalling through both TNFR1 and IFN γ R is essential for the non-specific effector phase against intracellular pathogens.

TNF α acts alone or synergistically with IFN- γ selectively to kill virus-infected cells and to protect uninfected cells from infection^{22,23}. To investigate the role of TNFR1 in mediating the anti-viral activity of TNF, we analysed the anti-viral response of *Tnfr1*⁰/*Tnfr1*⁰ mutants by measuring virus yield after infection. Replication of lymphocytic choriomeningitis virus (LCMV) is controlled in infected mutants and comparable to that in control mice (not shown). In addition, cytotoxic T-cell responses to vaccinia (WR strain) virus and LCMV²⁴ were within normal ranges (Fig. 3b). Moreover, the primary foot pad swelling reaction after local infection with LCMV was comparable in mutant and control mice, albeit delayed by one day in the mutant (Fig. 3c); the small delay might reflect enhanced compensatory IFN- γ levels. This indicates that expression of TNFR1 is essential neither for normal CTL induction nor for CTL effector function. Whether susceptibility to other viruses is changed in *Tnfr1*⁰/*Tnfr1*⁰ mice remains to be evaluated. A similar mutation of *Tnfr1* has recently been reported²⁵.

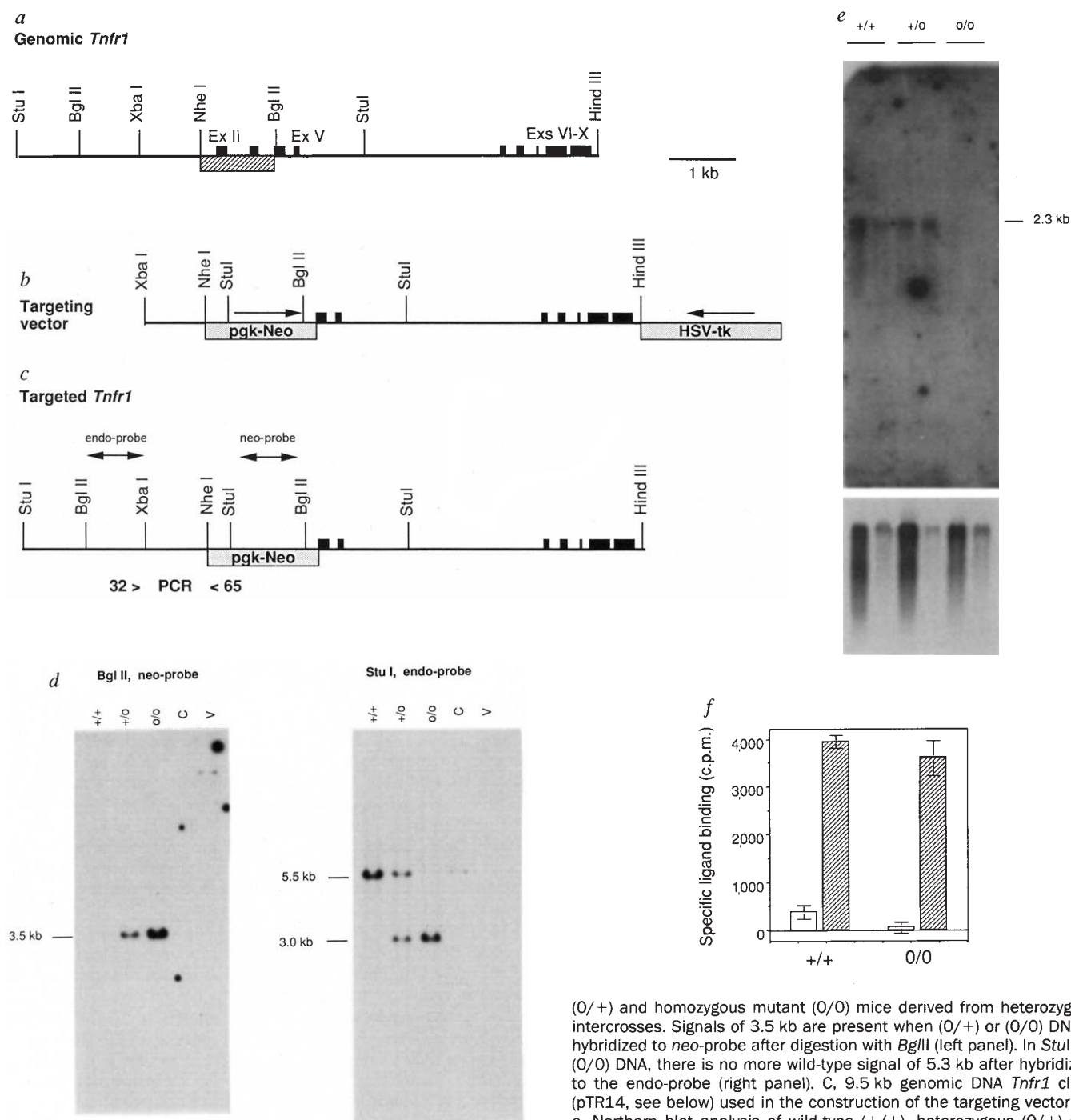


FIG. 1 Disruption of the *Tnfr1* gene by homologous recombination. **a**, Structure and partial restriction map of the wild-type mouse *Tnfr1* gene consisting of 10 exons (Ex)⁵. Exon I (not shown) is located ~7.7 kb upstream of exon II. **b**, Targeting vector used to generate mice lacking a functional *Tnfr1*. A genomic fragment containing the exons II and III and 5' sequences of exon IV (**a**, hatched region) was replaced by a neomycin resistance gene flanked by phosphoglycerate kinase-1 (*pgk-Neo*) promoter- and poly(A)⁺ signal sequences²⁶. The insertion of the *neo* cassette resulted in the introduction of new *Bgl*III and *Stu*I sites which were used to confirm targeting events. The *Tnfr1* homologies provided at both sides of the *neo* cassette were 868 bp and ~5 kb, respectively. For negative selection against random integrations, a herpes simplex virus thymidine kinase (*HSV-tk*) gene under the control of its own promoter was fused to the 3' homology. Arrows indicate the transcriptional directions. **c**, Predicted structure of the *Tnfr1* after targeted integration of the vector. Probes used for Southern blot analysis of mice are indicated, as well as the position of the primers used for PCR screening of ES cells. **d**, Southern blot analysis of wild-type (+/+), heterozygous

(0/+) and homozygous mutant (0/0) mice derived from heterozygous intercrosses. Signals of 3.5 kb are present when (0/+) or (0/0) DNA is hybridized to *neo*-probe after digestion with *Bgl*III (left panel). In *Stu*I cut (0/0) DNA, there is no more wild-type signal of 5.3 kb after hybridizing to the endo-probe (right panel). **C**, 9.5 kb genomic DNA *Tnfr1* clone (pTR14, see below) used in the construction of the targeting vector (**V**). **e**, Northern blot analysis of wild-type (+/+), heterozygous (0/+) and homozygous mutant (0/0) mice derived from heterozygous intercrosses. Upper panel, mRNA isolated from liver (left lane of each column) and spleen (right lane of each column) was hybridized with a *Spel*-*Bgl*III fragment extending from nucleotide 88 to nucleotide 338 of the previously published cDNA²⁷. Lower panel, the same blot rehybridized with a β -actin probe for comparison of the applied amounts of RNA. **f**, Binding of human and mouse TNF α to thymocytes from wild-type (+/+) and homozygous mutant (0/0) mice. Freshly isolated thymocytes were cultured overnight in RPMI medium containing 10% FCS and 20 μ g ml⁻¹ concanavalin A. Triplicates of 2.5×10^6 cells were incubated with 15 ng ml⁻¹ human ¹²⁵I-TNF α (open bars) or mouse ¹²⁵I-TNF α (hatched bars) as described previously²⁸. Non-specific binding was determined in the presence of a 200-fold excess of unlabelled ligand and subtracted from total binding. Recombinant human and mouse TNF α were labelled to a specific radioactivity of 6.2×10^7 c.p.m. mg⁻¹ and 8.9×10^7 c.p.m. mg⁻¹, respectively. Values are mean $\pm$ s.d.

METHODS. A 7 kb *Xba*I-*Hind*III DNA fragment from the 9.5 kb genomic DNA clone pTR14 (derived from mouse strain C57BL/6) was subcloned into pBluescript II KS (Stratagene). This subclone, pTR34, comprised all

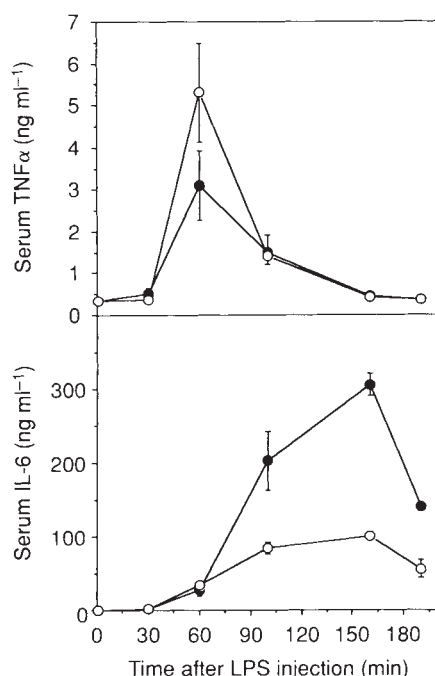


FIG. 2 Systemic release of cytokines after LPS challenge. Mice were injected with 50 μ g LPS (*S. enteritidis*) in 0.2 ml pyrogen-free 0.9% saline. Blood was taken at indicated time points and cytokine concentrations measured in triplicate. Serial dilutions of serum samples were assayed by ELISA for TNF α and IL-6 (Endogen Inc., Boston, USA) as recommended by the supplier. Absorbance values read at 450 nm were converted to concentrations (ng ml⁻¹) in the serum by comparison with the respective standard curve of each cytokine. Data were processed by non-parametric statistics and given as median values $\pm$ median deviation. Closed symbols: wild-type mice; open symbols: mutant mice.

transcribed sequences of the *Tnfr1* except exon I and parts of the 3' non-translated region. A 1,1158 bp fragment extending from a *NheI* site in intron 1 to a *BglII* site in exon IV (a, hatched region) was removed from pTR34, thereby excluding from the targeting vector $\sim$ 100 codons of exons II to IV including the splice acceptor of exon IV. The neomycin resistance cassette was cloned blunt ended between the filled *NheI* and *BglII* sites in the same transcriptional orientation as the *Tnfr1* gene (pTR34neo). The site for the integration of the *Clal*-*HindIII* restricted HSV-*tk* gene with its own promoter at the end of the 3' region of homology was generated by digestion of pTR34neo with *HindIII* and *Clal* (which cleaves in the polylinker). This resulted in a reversed transcriptional orientation of the HSV-*tk* gene as compared with the *Tnfr1* and *neo'* sequences. The targeting vector (30 μ g) was linearized with *XbaI* and electroporated at 250 V and 500 μ F into 3×10^7 E14 ES cells derived from mouse strain 129 (ref. 29). The cells were plated onto a layer of irradiated, G418-resistant primary mouse embryonic fibroblasts and subjected to G418 and FIAU selection for 12 days as described³⁰. The ratio of enrichment of targeted clones by positive-negative selection as compared to G418 selection alone was 1/3. Surviving clones were isolated and expanded for analysis and microinjection. 109 clones were screened by PCR and 6 ES cell clones were identified as correctly targeted (this is a frequency of 1/18). PCR-positive clones were confirmed by Southern blot analysis (d). Chimeric mice were generated by microinjection of ES cell clones carrying a mutated *Tnfr1* allele into C57BL/6 host blastocysts. Of the 21 mice born, 17 were males, from which 9 transmitted the mutated *Tnfr1* allele to their offspring.

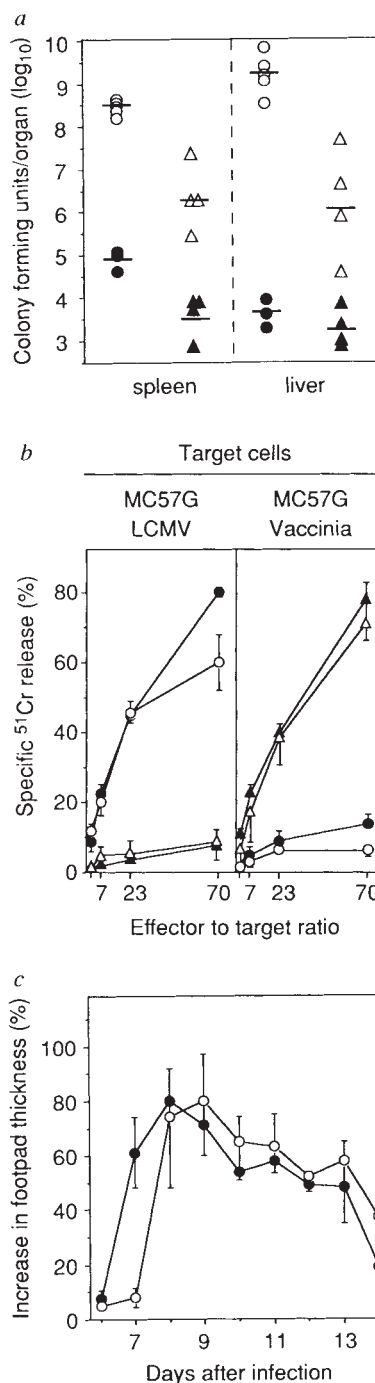


FIG. 3 Defence against bacterial and viral infections. a, Defence against infection by *Listeria monocytogenes*. Mice were infected intravenously with 4.0×10^3 (●) or 2.5×10^2 (▲) CFU of *L. monocytogenes*. Numbers of viable *L. monocytogenes* in spleens and livers of infected animals were determined 4 days after infection by plating serial 10-fold dilutions of organ homogenates in PBS on Trypticase-soy agar³¹. The experiment was repeated twice with similar results. Closed symbols: wild-type mice; open symbols: mutant mice. b, Cytotoxic T-cell responses. Mice were immunized by local infection with 3×10^3 plaque-forming units (PFU) of lymphocytic choriomeningitis virus (LCMV WE, ●) or by intravenous injection of 2×10^6 PFU of vaccinia virus (WR strain, ▲). T cells were isolated from spleens and assayed for specific lysis of MC57G(H-2^b) target cells infected either with LCMV or vaccinia virus. Values are mean of four individual measurements. Spontaneous lysis was below 19% in all experiments. Closed symbols: wild-type mice; open symbols: mutant mice. c, Specific immune response against LCMV. Thickness of footpads in wild-type mice (●) and mutant mice (○) after local infection with 3×10^3 PFU of LCMV WE are given relative to preinfection values³². Values are mean of individual measurements of four footpads.

In conclusion, the presented data indicate an essential role for TNFR1 in non-specific immunity against an intracellular bacterium, but not against the viruses tested. In addition, we have shown that the systemic toxicity of TNF α depends on signalling through TNFR1, but that during LPS-induced toxicity other components may also be activated, thus effectively bypassing TNFR1. □

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- Beutler, B. *Tumour Necrosis Factor: the Molecules and their Emerging Role in Medicine* (Raven, New York, 1992).
- Aggarwal, B. & Vilecek, J. *Tumour Necrosis Factors: Structure, Function, and Mechanism of Action* (Marcel Dekker, New York, 1992).
- Fiers, W. *FEBS Lett.* **285**, 199–212 (1991).
- Dembic, Z. et al. *Cytokine* **2**, 231–237 (1990).
- Rothe, J., Bluethmann, H., Gentz, R., Lesslauer, W. & Steinmetz, M. *Molec. Immun.* **30**, 165–175 (1993).
- Banner, D. W. et al. *Cell* **73**, 1–20 (1993).
- Lewis, M. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 2830–2834 (1991).
- Bone, R. C. et al. *Chest* **101**, 1644–1655 (1992).
- Galanos, C., Freudenberg, M. A. & Reutter, W. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5939–5943 (1979).
- Freudenberg, M. A. & Galanos, C. *Infect. Immun.* **59**, 2110–2115 (1993).
- Lesslauer, W. et al. *Eur. J. Immun.* **21**, 2883–2886 (1991).
- Ashkenazi, A. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 10535–10539 (1991).

- Waage, A. & Espevik, T. *J. exp. Med.* **169**, 1987–1992 (1988).
- Brouckaert, P., Libert, C., Everaerd, B. & Fiers, W. *Lymph. Cyt. Res.* **11**, 193–196 (1992).
- Waage, A. et al. *J. exp. Med.* **170**, 1859–1867 (1989).
- Shalaby, R., Waage, A., Aarden, L. & Espevik, T. *Clin. Immun. Immunopath.* **53**, 488–498 (1989).
- Fong, Y. et al. *J. exp. Med.* **170**, 1627–1633 (1989).
- Havell, E. A. *J. Immun.* **139**, 4225–4231 (1988).
- Nakane, A., Minagawa, T. & Kato, K. *Infect. Immun.* **56**, 2563–2569 (1988).
- Hauser, T., Frei, K., Zinkernagel, R. M. & Leist, T. P. *Med. microbiol. Immun.* **179**, 95–104 (1990).
- Huang, S. et al. *Science* **259**, 1742–1745 (1993).
- Wong, G. H. & Goeddel, D. V. *Nature* **323**, 819–822 (1986).
- Feduchi, E., Alonso, M. A. & Carrasco, L. J. *Virology* **63**, 1354–1389 (1989).
- Zinkernagel, R. M., Rüedi, E., Althage, A., Hengartner, H. & Reimann, G. *J. exp. Med.* **168**, 1187–1192 (1988).
- Pfeffer, K. et al. *Cell* **73**, 457–467 (1993).
- Adra, C. N., Boer, P. H. & McBurney, M. W. *Gene* **60**, 65–74 (1987).
- Rothe, J. G., Brockhaus, M., Gentz, R. & Lesslauer, W. *Immunogenetics* **34**, 338–340 (1991).
- Gehr, G., Gentz, R., Brockhaus, M., Loetscher, H. R. & Lesslauer, W. *J. Immun.* **149**, 911–917 (1992).
- Doetschmann, T. et al. *Nature* **330**, 576–578 (1987).
- Köntgen, F., Süss, G., Stewart, C., Steinmetz, M. & Bluethmann, H. *Intern. Immun.* (in the press).
- Biandini, R. V. & Langman, R. E. *Scand. J. Immun.* **1**, 379–391 (1972).
- Leist, T. P., Eppler, M. & Zinkernagel, R. M. *J. Virology* **63**, 2813–2819 (1989).

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Primary structure and functional expression of a rat G-protein-coupled muscarinic potassium channel

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PARASYMPATHETIC nerve stimulation causes slowing of the heart rate by activation of muscarinic receptors and the subsequent opening of muscarinic K⁺ channels in the sinoatrial node and atrium^{1–4}. This inwardly rectifying K⁺ channel is coupled directly with G protein^{5–10}. Based on sequence homology with cloned inwardly rectifying K⁺ channels, ROMK1 (ref. 11) and IRK1 (ref. 12), we have isolated a complementary DNA for a G-protein-coupled inwardly rectifying K⁺ channel (GIRK1) from rat heart. The GIRK1 channel probably corresponds to the muscarinic K⁺ channel because (1) its functional properties resemble those of the atrial muscarinic K⁺ channel and (2) its messenger RNA is much more abundant in the atrium than in the ventricle. In addition, GIRK1 mRNA is expressed not only in the heart but also in the brain.

Because the muscarinic K⁺ channel is expressed in the heart and shows inward rectification properties similar to those of ROMK1 and IRK1, we screened a rat heart cDNA library for clones with sequence similarity to these K⁺ channel cDNAs (see Fig. 1 legend). A cDNA clone of about 4.2 kilobases (kb), GIRK1 (Fig. 1a), was isolated and found to encode a protein that is structurally similar to ROMK1¹¹ and IRK1¹² with two transmembrane regions (M1, M2) and a putative pore region (H5). GIRK1 shares 43% identity with IRK1 and 39% with ROMK1 in the total amino-acid sequence (Fig. 1b). The cRNA for GIRK1, when injected into *Xenopus* oocytes, gave rise to functional K⁺ channels with electrophysiological properties that closely resemble those of the G-protein-coupled muscarinic K⁺ channel from the heart.

As with the muscarinic K⁺ channel in the heart, the GIRK1

channel could be activated by muscarinic receptor activation. Following coinjection of m2 muscarinic receptor cRNA¹³ and GIRK1 cRNA, bath application of 1 μ M carbachol induced an inwardly rectifying current (Fig. 2a, b). The time course of the induced currents, obtained by subtracting the currents before from those after bath application of carbachol, showed slow activation at the beginning of voltage pulses (Fig. 2a, b), a characteristic property of muscarinic K⁺ channels³. This carbachol-induced inwardly rectifying current was not observed when either the m2 muscarinic receptor ($n=4$) or the GIRK1 ($n=4$) cRNA was injected. As the m2 muscarinic receptor activates G proteins¹⁴, the activation of GIRK1 channel was probably mediated by endogenous oocyte G proteins¹⁵ that were coupled with the m2 receptor.

Because the identity of the endogenous G proteins that coupled m2 muscarinic receptors to GIRK1 channels could not be readily determined, we investigated the effect of an exogenously introduced G-protein trimer, composed of α_{i2} (ref. 16), β_1 (ref. 17) and γ_2 (ref. 18) subunits, on the GIRK1 channel. Previous studies suggested that the muscarinic K⁺ channel is coupled with $G\alpha_i$ (refs 7–9) and/or $\beta\gamma$ (refs 6, 10) subunits, and that a basal level of muscarinic K⁺ channel activation can be observed without activating muscarinic receptors in the atrial cell¹⁹. When cRNA for m2 receptor, $G\alpha_{i2}$, β_1 , γ_2 and GIRK1 were coinjected into oocytes, an inwardly rectifying current, similar to the carbachol-induced current, was observed without activating the m2 muscarinic receptor. Activation of m2 receptor did not significantly increase this current. The large basal activity and the lack of coupling between the exogenously introduced muscarinic receptors and $G\alpha_{i2}$, β_1 , γ_2 might be explained by the inappropriate combination or ratio of the injected G-protein subunits in a heterologous expression system. The inwardly rectifying current was also observed in oocytes injected with $G\alpha_{i2}$, β_1 , γ_2 and GIRK1 cRNA, but not with $G\alpha_{i2}$, β_1 , γ_2 or GIRK1 cRNA alone (Fig. 2c, d). These results suggest that GIRK1 channels are activated by exogenously introduced G-protein subunits.

As expected for a K⁺ selective inwardly rectifying channel³, the activation potential of GIRK1 channel (see legend to Fig. 2g) shifted to hyperpolarized potential upon reduction of extracellular K⁺ concentration (Fig. 2e, f) and the amount of shift follows the change in the K⁺ equilibrium potential (E_K) as predicted by the Nernst equation (Fig. 2g).

To test whether the activation of GIRK1 is caused by direct interaction with G proteins, we examined the effect of the non-hydrolysable GTP analogue, GTP- γ S, applied to the cytoplasmic side of excised patches. In cell-attached recordings from

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FIG. 1 a, Nucleotide and deduced amino-acid sequence of GIRK1. The GIRK1 cDNA was sequenced on both strands using Sequenase (USB corp.) Nucleotide A of the first methionine codon (ATG) is assigned as +1. The IRK1 clone has ~4.2 kb, with only one long open reading frame, predicting a protein of 501 amino acids. There is no stop codon in the cDNA sequence 5' to the first methionine codon. Although we have analysed ≥ 5 independent cDNA clones from one heart and two brain cDNA libraries and >20 RACE PCR (rapid amplification of cDNA ends polymerase chain reaction³²) products using two different reverse transcriptases (Invitrogen, and Gibco BRL), we could extend this cDNA sequence by only 19 bases (5' CCTATTTGGTGCTGGTTTG) at the 5' end. However, a hamster GIRK1 cDNA has been isolated with more sequences at the 5' end. Multiple stop codons are present about 230 bp upstream of the methionine at position +1 and there are no other methionine codons in this region, indicating that the sequences shown in this figure include the entire protein-coding sequence. Only part of the 3' untranslated sequence is shown. The proposed transmembrane segments M1 and M2 are boxed as is a segment with sequence similarity to the H5 region of voltage-gated K⁺ channels. There are 9 potential protein kinase C phosphorylation sites (T64, S203, S284, S357, S396, T407, S424, T455 and S497). **b**, Alignment of amino-acid sequences (single-letter code) of GIRK1, the inward rectifier K⁺ channel (IRK1)¹² and the ATP-regulated K⁺ channel (ROMK1)¹¹. -, Amino acids identical to those in GIRK1. -, Gaps introduced to improve alignment. The proposed transmembrane regions M1 and M2 and the H5-like segment are boxed. The IRK1 amino-acid sequence in ref. 12 contains one error; the residue at position 53 is His, rather than Arg. This error has been corrected in this alignment as well as in the entry to the EMBL Data Library. **METHODS**. Molecular cloning: PCR and screening of cDNA libraries were as described before³³. Two degenerative primers were designed for the published amino-acid sequences KDGRNCVQ (IRK1 amino acids 50–57) and VFQSVG (IRK1 amino acids 162–168)¹². The sequences of the primers are AA(A or G)GAIGGIC-GITG(C or T)AA(C or T)(C or A)T and ICCIAC-IATIGA(T or C)TG(G or A)AAIA.

		5'..... CAGCGCTTGACCTCTGCGCTCCGCTTCGTGTTTGAATCTGGATCTCCCTCCGTATT		-1
a	Met Ser Ala Leu Arg Arg Lys Phe Gly Asp Asp Tyr Gln Val Val Thr Thr Ser Ser Ser Gly Ser Gly Leu Gln Pro Gln Gly Pro Gly	10	20	30
	ATG TCT GCA CTC CGA AGG AAA TTT GGG GAC GAT TAC CAG GTA GTG ACC ACT TCG TCC AGC GGT TCG GTG CAG CCC CAG GGG CCA GGA	40	50	60
	Gln Gly Pro Gln Gln Gln Val Val Pro Lys Lys Lys Arg Gln Arg Phe Val Asp Lys Asn Gly Cys Asn Val Gln His Gly Asn Leu	70	80	90
	CAG GGC CCA CAG CAG CAG CTT GTA CCC AAG AAG AAA CCG CAG CCG TTC GTG GAC AAG AAC GGT CCG TGC AAT GTG CAG CAC GGC AAC CTG	100	110	120
	Gly Ser Glu Thr Ser Arg Tyr Leu Ser Asp Leu Phe Thr Thr Leu Val Asp Leu Lys Trp Arg Trp Asn Leu Phe	130	140	150
	GGC AGC GAG ACC AGT CCG TAC CTT TCC GAC CTC TTC ACT ACC CTG GTG GAT CTC AAG TCG CGT TCG AAC CTC TTT	160	170	180
	Tyr Thr Val Ala Trp Leu Phe Met Ala Ser Met Trp Trp Val Ile Ala Tyr	190	200	210
	TAC ACC GTG GGC TGG CTC TTC ATG GCG TCC ATG TGG TGG ATC GCT TAT ACC CCG	220	230	240
	Thr Pro Cys Val Ala Asn Val Tyr Asn Phe Pro Ser	250	260	270
	ACT CCC TGT GTG GCC AAT GTC TAT AAC TTC CCC TCC	280	290	300
	Ile Thr Asp Lys Cys Pro Glu	310	320	330
	ATC ACC GAC AAG TGC CCC GAG GGC ATC ATC CTT TTC CTT TTC CAG TCC ATC CTT GGC TCC ATC GTG GAC GCT TTC CTC ATC GGC TGC	340	350	360
	Phe Ile Lys Met Ser Gln Pro Lys Lys Arg Ala Glu Thr Leu Met Phe Ser Glu His Thr Leu Ser Met Arg Asp Gly Lys Leu Thr	370	380	390
	TTC ATC AAG ATG TCC CAG CCC AAA AAG CCG GGC GAG ACC CTC ATG TTT AGC GAG CAT GCG GTT ATT TCC ATG AGG GAC GGA AAA CTC ATC	400	410	420
	Leu Met Phe Arg Val Gly Asn Leu Arg Asn Ser His Met Val Ser Ala Gln Ile Arg Cys Lys Leu Leu Lys Ser Arg Gln Thr Pro Glu	430	440	450
	CTC ATG TTC CCG GTG GGC AAC CTC CCG AAC CAC ATG GTC TCC GCG CAG ATC CCG TCC AAG CTG CTC AAA TCT CCG CAG ACA CCG GAG	460	470	480
Gly Glu Phe Leu Pro Leu Asp Gln Leu Glu Leu Asp Val Gly Phe Ser Thr Gly Ala Asp Gln Leu Phe Leu Val Ser Pro Leu Thr Ile	490	500	510	
GGT GAG TTT CTA CCC CTT GAC CAA CTT GAA CTG GAT GTA GGT TTT AGT ACA GGG GCA CAT CAA CTT TTT CTT GTG TCC OCT CTC ACC ATT	520	530	540	
Cys His Val Ile Asp Ala Lys Ser Pro Phe Thr Asp Leu Ser Gln Arg Ser Met Gln Thr Glu Gln Phe Glu Val Val Ile Leu Glu	550	560	570	
TGC CAC GTG ATT GAT GGC AAA AGC CCG TTT TAT GAG CTA TCC CAG CGA AGT GCA ACT GAA CAG TTC GAG GTG GAT GTC ATC CTG GAA	580	590	600	
Gly Ile Val Glu Thr Thr Gly Met Thr Cys Gln Ala Arg Thr Ser Tyr Thr Glu Asp Glu Val Leu Trp Gly His Arg Phe Phe Pro Val	610	620	630	
GGC ATC GTG GAA ACC ACA GGG ATG ACT TGT CAA GCT CGA ACA TCA TAC ACC GAA GAT GAA GTT CTT TGG GGT CAT CTT TTT TTC OCT GTA	640	650	660	
Ile Ser Leu Glu Glu Gly Phe Phe Lys Val Asp Tyr Ser Gln Phe His Ala Thr Phe	670	680	690	
ATT TCT TTA GAA GAA GGA TTT AAA GTC GAT TAC TCC CAG TTC CAT GCA ACC TTT GAA GTC CCC ACC CCG TAC Ser Val Lys Glu	700	710	720	
Gln Glu Glu Met Leu Leu Met Ser Ser Pro Leu Ile Ala Pro Ala Ile Thr Asn Ser Lys Glu Arg His Asn Ser Val Glu Cys Leu Asp	730	740	750	
CAG GAA GAA ATG CTT CTC ATG TCT TCC OCT TTA ATA GCA CCA CCA GGC ATA ACC AAC AGC AAA GAA AGA CAC AAT TCT GTG GAG TGC TTA GAT	760	770	780	
Gly Leu Asp Asp Ile Ser Thr Lys Leu Pro Ser Lys Leu Gln Lys Ile Thr Gly Arg Asp Asp Phe Pro Lys Lys Leu Arg Met Ser	790	800	810	
GGA CTA GAT GAT ATT AGC ACA AAA CTT CCA TCG AAG CTG CAG AAA ATT ACG GGG AGA GAA GAT TTT CCC AAA AUA CTT CTG AGG ATG AGT	820	830	840	
Ser Thr Thr Ser Glu Lys Ala Tyr Ser Leu Gly Asp Leu Pro Met Lys Leu Gln Arg Ile Ser Val Pro Gly Asn Ser Glu Glu Lys	850	860	870	
TCT ACA ACT TCA GAA AAA GGC TAT AGT TTG GGT GAT TTG CCC ATG AAA CTC CAA CGA ATA AGT TGT GGT OCT GGC AAC TCT GAA GAA AAA	880	890	900	
Leu Val Ser Lys Thr Thr Lys Met Ser Ser Pro Met Ser Gln Ser Val Ala Asp Leu Pro Pro Lys Leu Gln Lys Met Ala Gly Gly	910	920	930	
CTG GTA TCT AAA ACC ACC AAG ATG TTA TCA GAT CCC ATG ACG CAG TCT GTG GGC GAT TTG CGA CCG AAG CTT CAA AAG ATG GCT GGA GGA	940	950	960	
Pro Thr Arg Met Glu Gly Asn Leu Pro Ala Lys Leu Arg Lys Met Asn Ser Asp Arg Phe Thr	970	980	990	
CCT ACC AGG ATG GAA GGG AAT CTT CCA GGC AAA CTA AGA AAA ATG AAC TCT GAC CCG TTC ACA TAG CAAACACCCCATAGGACATTATTTCATGTTTGG	1000	1010	1020	
ATTATGTTTATGTCACATTTTGGCTGATAAGATTAATCTCTCCGGAATCTGAGAGCTTATCCAGTCTGCGAAATTCACAGAGACTCTTCATTTGAGTGTCTGTATGCTGTGACATGAGTT	1030	1040	1050	
ACAAAGGAGGACATCATAGAAAGCTAATGATTTGGCATGTATATATCATACCAAGCATGCAATAATGTGCAAAATTTTGCATTTATGTTTTCCTGCATGATT.....3	1060	1070	1080	
b	GIRK1	MSALRRKFGDDYQVVTTSSSGSLQ-----PQGPQGQPQQQLVPKKKR	43	
	IRK1	:GSV:---TNR:SI:SEED:MK:ATMA---VAN:F:N:KSKVHTRQQC:	44	
	ROMK1	:G:SE:SV---FR:LIRALTERMFKHLRRWFTHIF:RSR:-----	39	
	GIRK1	QRFVDKNGKCNVQHGNLGSSETS-RLSLDLFTTLVDLKWRLNLF	92	
	IRK1	S:::K:D:H:::FI:V:EKGQ:::A:I:::C::IR:::M:V:::C:AFV	93	
	ROMK1	A:L:S:E:::IEF::VDAQSRFIFV:IW::VL:::YKMTV::TAFL	89	
	GIRK1	VAWLFFMASMWWVIAITRGDLNKA-HVGNYPVCVANVYNFPFS	141	
	IRK1	LS:::FGCVF:L::LH:::DTS-K:SK--A::SE:NS:TA:::S:::Q	140	
	ROMK1	GS:FLFGLL:Y:V::VHK::PEFYPPD:R:::E:INGMT:::SL:::Q	139	
	GIRK1	ATIGYGYR:YITDKCP:EGILFLFQSIILGSIVDAFLIGCMFIKMSQPKKRA	191	
	IRK1	T:::F:CV::E:I:AVFMVV:::V:C:I:::I:AVMA:AK:::N	190	
	ROMK1	V:::F:EV:EQ:AT:A:F:LI:::V:INS:M:A:ILA:I:R:::189		
	GIRK1	ETLMFSEHAVISMEDGKLTLMFRVGNLRNSHMSVSAQIRCKLLKSRTPEG	241	
	IRK1	:::V::HN:::A:::C::W:::K:L:E:HV:AQ:::I:S::	240	
	ROMK1	K:IT::KN:::K:G:::C:LI:::A:::K:LLIGSHIYG:::TTI:::239		
	GIRK1	EFLPLDQLELDVGFSTGADQLFLVSPLTICHVIDAKSPFYDLSQRSMQTE	291	
IRK1	:YI:::IDIN:::DS:I:RI:::I::V:E:ED::L:::KQDIDNA	290		
ROMK1	:TII:::TNINPVVDA:NEN::FI:::Y:I::HN:::FHMAAETLSQQ	289		
GIRK1	QFEVVVILEGIVETTGMTQCARTSYTEDEVWGRFFPVISLEE-GFFKV	340		
IRK1	D::I:::M::A:A::T:C:S::LAN:I:::YE:LFE:K-HYY::	339		
ROMK1	D::L:::F:D:T:S:SA:::V:::VPE:::Y::V:IV:KTE:KYR::339			
GIRK1	DYSQFHATFEVPTPPYSVKEQEEMLLMSSPLIAPAITNSKERHNSVECLD	390		
IRK1	:::R::K:Y:::NT:LCSARD---:AEKKYILSNANSF-----:YE	377		
ROMK1	:FHN:GK:V:::ET:HCAM-----:Y	360		
GIRK1	GLDDISTKLPSKLLQKITGREDFPKKLLRMSSTTSEKAYSLGDLPMKLQRI	440		
IRK1	--NEVA--:T::EEEEEDSENGV:E-----:STDS--PP:--ID:HNQ	412		
ROMK1	NEK:ARARMK-----:RG:DNPNFVL-----380			
GIRK1	SSVPGNSEKKLVSKTTKMLSDPMSQSVADLPKLLQKMAGGPTRMEGNLPA	490		
IRK1	A:::E:::R-----:L:R:SEI--	428		
ROMK1	:E:DETDDTQM-----	391		
GIRK1	KLRKMNSDRFT 501			
IRK1				
ROMK1				

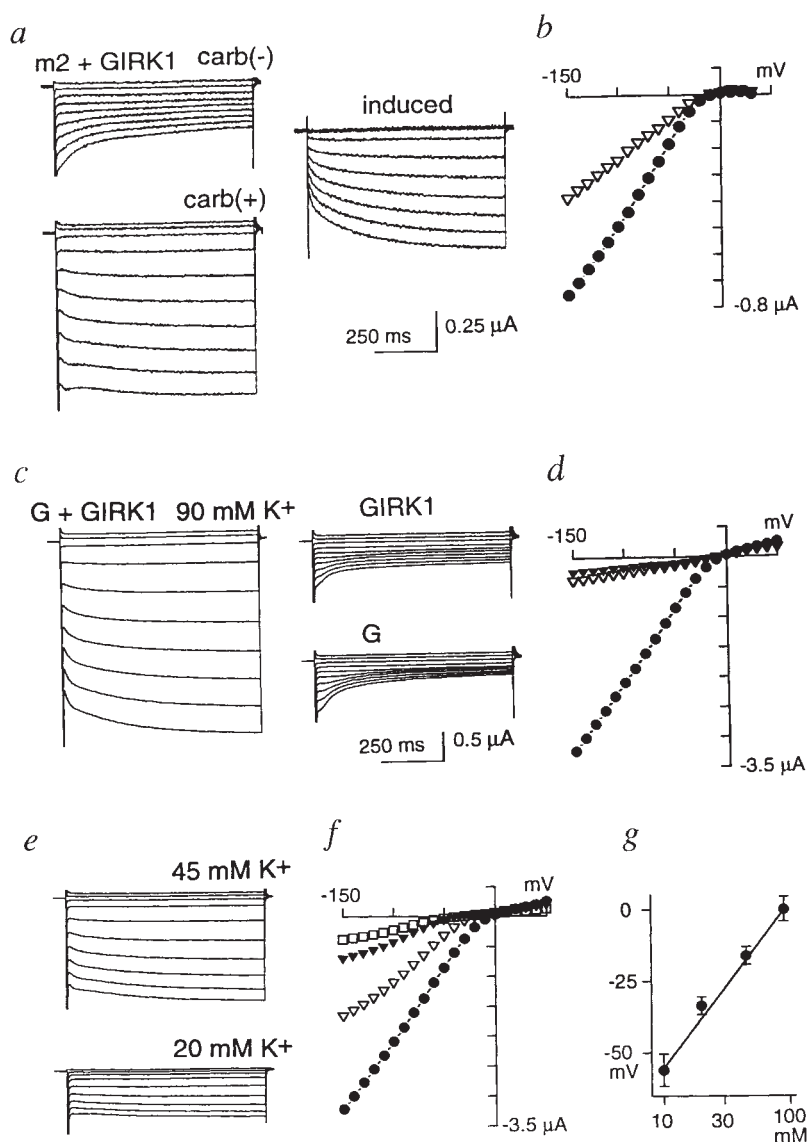
oocytes injected with GIRK1 and $G\alpha_{i2}$, β_1 , γ_2 cRNA, single-channel openings were observed in the absence of activated m2 receptor, as was found for recordings under two-electrode voltage-clamp. The single-channel activity decreased after excising the membrane patch into a solution containing no GTP, but could be restored by applying 100 μ M GTP- γ S to the cytoplasmic side of the membrane (Fig. 3a). In five patches, the normalized open probability (P_0) decreased by sixfold after excising into a GTP-free solution, and increased by 10-fold after applying GTP- γ S (Fig. 3b and legend). The channel activities recorded in the cell-attached configuration and those recorded in the presence of cytoplasmic GTP- γ S appeared to come from the same channel, because the single-channel conductance (42 pS) and the kinetics were similar in these two conditions (see legend to Fig. 3a). These results imply that the activation of GIRK1 is caused by an interaction with G proteins that are associated with the patch of membrane, rather than diffusible

second messengers that are activated by G proteins.

To see if the expressed GIRK1 channel is similar to the muscarinic K^+ channel in the heart^{3,20}, we measured the single-channel conductance and the open time and examined if the inward rectification of GIRK1 channel depended on internal Mg^{2+} , as has been shown for the muscarinic K^+ channel²⁰. GIRK1 showed a single-channel conductance of 42 pS in 170 mM K^+ (Fig. 3c, e), as compared with 41 pS in 150 mM K^+ for the muscarinic K^+ channel²⁰. The open time distribution was similar to that reported for the muscarinic K^+ channel (see legend to Fig. 3a). Finally, GIRK1 channels activated by GTP- γ S showed strong inward rectification which depended on the presence of internal Mg^{2+} ; when Mg^{2+} was removed from the bath solution, outward currents were recorded from the inside-out patches (Fig. 3d, e). All of the single-channel recordings were obtained in the presence of 2.5 mM cytoplasmic ATP which blocks ATP-sensitive K^+ channels²¹. Taken together, these results suggest

FIG. 2 Inwardly rectifying K^+ currents recorded by two-electrode voltage clamp from *Xenopus* oocytes injected with GIRK1 cRNA. a, Current traces recorded in 90 mM K^+ solution from oocytes injected with m2 muscarinic receptor and GIRK1 cRNA before and after application of 1 μ M carbachol. The carbachol-induced currents are the difference of the former two sets of traces. The holding potential was 0 mV, and current traces elicited by steps to +50, +20, -10, -40, -70, -100 and -130 mV are shown. The amplitude of carbachol-induced current at -150 mV ranged from 290–760 nA ($n=8$). b, Current-voltage (I - V) plot of the carbachol-induced current traces in a. Current amplitude at the beginning (∇) and at the end ($\bullet$) of the steps were plotted. c, Current traces recorded in 90 mM K^+ solution from oocytes injected with both GIRK1 and $G\alpha_{i2}$, β_1 , γ_2 cRNA or with either GIRK1 or $G\alpha_{i2}$, β_1 , γ_2 cRNA alone. The holding potential and the depolarization steps are as in a. The amplitude of the GIRK1 current at -150 mV after linear-leak subtraction ranged from 600–2,700 nA ($n=35$). d, I - V plot of the current traces in c. Current amplitude at the end of the steps were plotted. Symbols are ($\bullet$) both GIRK1 and $G\alpha_{i2}$, β_1 , γ_2 cRNA, (∇) only GIRK1 cRNA and ($\blacktriangledown$) only $G\alpha_{i2}$, β_1 , γ_2 cRNA. e, Current traces recorded in 45 mM K^+ or 20 mM K^+ solution from the same oocytes injected with both GIRK1 and $G\alpha_{i2}$, β_1 , γ_2 cRNA as in c. The holding potential and the depolarization steps are as in a. f, I - V plot of the inwardly rectifying current from oocytes injected with GIRK1 and $G\alpha_{i2}$, β_1 , γ_2 in various external K^+ concentration. Symbols are: ($\bullet$), 90 mM K^+ ; (∇), 45 mM K^+ ; ($\blacktriangledown$), 20 mM K^+ ; ($\square$), 10 mM K^+ . Current amplitude at the end of the steps were plotted. E_K s in these solutions predicted by the Nernst equation were 0 mV (90 mM K^+), -17 mV (45 mM K^+), -37 mV (20 mM K^+), and -55 mV (10 mM K^+). (The intracellular K^+ concentration of oocytes was assumed to be 90 mM (ref. 15).) The small outward currents in c–f include some endogenous currents of the oocyte. g, Logarithmic plot of extracellular K^+ concentration versus activation potential (the potential where the slope of current-voltage relation starts to increase). Vertical bars show standard deviation. The mean and standard deviation values are 0 ± 4 ($n=9$; 90 mM), -16 ± 3 ($n=5$; 45 mM), -33 ± 3 ($n=7$; 20 mM) and -56 ± 6 ($n=2$; 10 mM). The straight line shows the equilibrium potential of K^+ predicted by the Nernst equation.

METHODS. Oocytes were injected with 50 nl cRNA solution, which contains ~ 300 ng ml^{-1} GIRK1 cRNA and 300 ng ml^{-1} m2 muscarinic receptor cRNA (a, b), or either, or both 500 ng ml^{-1} GIRK1 cRNA and 15 ng ml^{-1} each of $G\alpha_{i2}$, β_1 and γ_2 cRNA (c, d, e, f, g). Electrophysiological recordings were done 48–96 h later at 22–25 $^{\circ}C$ by two-electrode



voltage clamps³³. Data acquisition and analysis were done on a computer using the pClamp program and a TL-1 A/D converter (Axon Instruments). Microelectrodes were filled with 3 M KCl and resistances were 0.7–1.5 M Ω . The bath solution contained 90 mM KCl, 3 mM $MgCl_2$, 5 mM HEPES (pH 7.4) (the 90 mM K^+ solution). In solutions with reduced K^+ concentration, K^+ was replaced with *N*-methyl glucamine.

that the GIRK1 channel corresponds to the muscarinic K^+ channel.

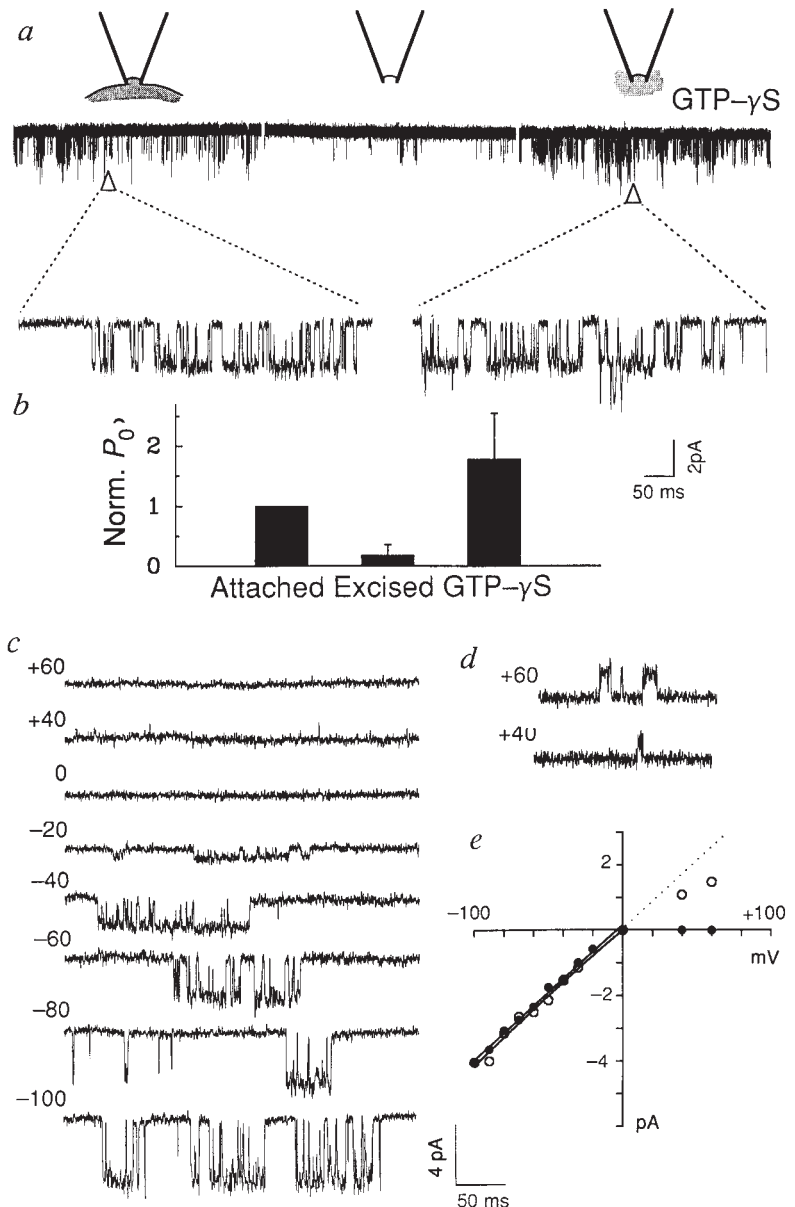
By northern analysis, we found that GIRK1 mRNA (about 4.5 kb and 6.0 kb) is more abundant in the heart atrium than in the ventricle (Fig. 4a, b), consistent with the distribution of the muscarinic K^+ channels determined by electrophysiological studies^{3,5,7}. GIRK1 mRNA was also detected in forebrain and

cerebellum, but not in liver or skeletal muscle (Fig. 4a). G-protein-coupled K^+ channels have been found in the brain^{22,23} and they are activated by various neurotransmitters, such as substance P²⁴, GABA^{25,26}, somatostatin^{27,28}, opioid^{29,30} and acetylcholine³¹. Thus, GIRK1 channel may play a role in regulating the neuronal activity in the brain as well as the excitability of the heart.

FIG. 3 Single-channel recordings from oocytes injected with GIRK1 and $G\alpha_{12}$, β_1 , γ_2 cRNA. **a**, **b**, GIRK1 channel is activated by cytoplasmic GTP- γ S. **a**, One-min segments of continuous recordings from a membrane patch initially in cell-attached configuration (left), subsequently excised (inside-out) into solution without GTP (middle), and then exposed to 100 μ M GTP- γ S (right). Expanded traces from the small segments indicated by triangles are shown below. Holding potential was -60 mV. Open time histograms were fitted by least squares with three exponentials. The mean and standard deviation ($n = 4$) of the three time constants and percentage contributions were: cell attached, 0.26 ± 0.11 ms ($21 \pm 8\%$), 1.2 ± 0.3 ms ($43 \pm 9\%$) and 7.2 ± 1.0 ms ($36 \pm 10\%$); excised plus GTP- γ S, 0.45 ± 0.35 ms ($25 \pm 13\%$), 2.3 ± 0.9 ms ($50 \pm 11\%$) and 8.9 ± 4.3 ms ($25 \pm 13\%$). The time constant of the muscarinic K^+ channel was 0.96 ms (ref. 34)– 1.4 ms (ref. 3). **b**, Open probability measured from continuous single-channel recordings during cell-attached, excised and GTP- γ S applied periods. Because the number of channels in the patch was not known, the open probability was normalized to the P_0 measured in cell-attached patches. The error bars show standard deviation. The normalized P_0 ($n = 5$) was 0.18 ± 0.18 after excising and 1.78 ± 0.76 with GTP- γ S (mean $\pm$ s.d.). The decrease on excision and the increase after exposure to GTP- γ S were significant by Student's paired t -test ($P < 0.05$). **c**, **d**, **e**, Single-channel conductance and the effect of removal of cytoplasmic Mg^{2+} on inward rectification. Holding potentials (mV) are given to the left of the traces. **c**, Recording of single-channel activity in the presence of cytoplasmic Mg^{2+} . The single-channel conductance was 42 ± 1 pS ($n = 5$). Free Mg^{2+} concentration in the cytoplasmic solution was calculated to be 2.5 mM. **d**, Recordings in Mg^{2+} -free cytoplasmic solution. Only outward channel events are shown. The inward single-channel conductance was 43 ± 2 pS ($n = 2$) in Mg^{2+} -free solution. **e**, I - V plots of single-channel current from the experiment in **c** ($\bullet$) and **d** ($\circ$). Straight lines show the best-fit having a slope conductance of 42 pS for Mg^{2+} and 43 pS for Mg^{2+} -free solution. The deviation of the outward current amplitude in Mg^{2+} -free solution from the ohmic conductance (dashed line) could be due to residual Mg^{2+} , which remains near the excised patch of membrane even after perfusing the bath with Mg^{2+} -free solution.

METHODS. Oocytes were injected with 50 nl cRNA, which contains ~ 300 ng ml^{-1} GIRK1 channel cRNA, 300 ng ml^{-1} m2 muscarinic receptor cRNA, and 10 ng ml^{-1} each of $G\alpha_{12}$, β_1 , γ_2 cRNA, or 500 ng ml^{-1} GIRK1 channel cRNA and 15 ng ml^{-1} each of $G\alpha_{12}$, β_1 , γ_2 cRNA. The channel activities were observed in the absence of carbachol; similar results have been obtained from oocytes injected with GIRK1 and $G\alpha_{12}$, β_1 , γ_2 cRNA, with or without m2 receptor cRNA. Single-channel activity was recorded in cell-attached or inside-out patches. Currents were recorded with a List EPC7 amplifier at 22 – 25° C. The continuous recordings were stored on VCR, transferred to disc at 2 – 8 kHz through an A/D converter (Instrutech), digitally filtered at 0.5 – 2 kHz and analysed by the FETCHAN program. Patch pipettes had resistances of 1 – 4 M Ω . The pipette solution (external) was 75 mM K_2SO_4 , 15 mM KCl, 2 mM $MgSO_4$, 10 μ M GdCl₃, 5 mM KOH and 10 mM HEPES (pH 7.4). GdCl₃ was included to suppress stretch channel activity³⁵. GdCl₃ was found to have little effect on the GIRK1 current recorded by two-electrode voltage clamp. The Mg^{2+} -containing bath

solution (internal) was 72.5 mM K_2SO_4 , 15 mM KCl, 4.4 mM $MgSO_4$, 2.5 mM K_2 -ATP, 5 mM KOH, 5 mM HEPES (pH 7.2). Total K^+ concentration was 170 mM, and free Mg^{2+} concentration was calculated to be 2.5 mM. The Mg^{2+} -free bath solution (internal) was 59 mM K_2SO_4 , 15 mM KCl, 10 mM K_2 -EDTA, 12 mM KOH, 2.5 mM K_2 -ATP, 5 mM HEPES (pH 7.2). For both bath solutions, a high concentration of ATP (2.5 mM) was used to suppress ATP-sensitive channels²¹. In cell-attached mode, the membrane potential of the patch differs from the applied potential (the potential difference between the pipette solution and the bath solution, each containing 170 mM K^+) by an amount equivalent to the resting potential of the oocyte which is close to E_K . Under this condition the applied potential is roughly the K^+ driving force across the patch membrane in both cell-attached mode and inside-out excised patch mode, so that the same channel is expected to exhibit the same single-channel current before and after excision of the membrane patch.



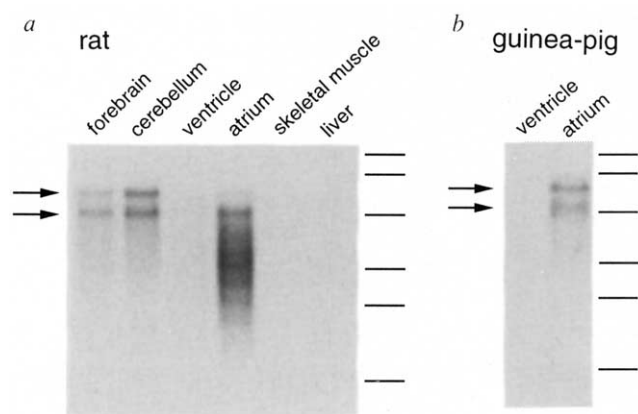


FIG. 4 Distribution of GIRK1 mRNA in various tissues. *a, b*, Northern blot analysis of GIRK1 mRNA expression. The lanes represent poly(A)⁺ RNA from *a*, rat forebrain, cerebellum, heart ventricle, atrium, skeletal muscle and liver, and from *b*, guinea-pig heart ventricle and atrium. Forebrain includes cerebral cortex, hippocampus, basal ganglia, thalamus, hypothalamus and olfactory bulb. Atrium preparation includes part of the vessel stems. Probes used are *a*, 4.2 kb cDNA of GIRK1 or *b*, 363 bp PCR fragment, obtained from cDNAs reverse-transcribed from guinea-pig heart RNA, which encodes identical amino acids to those of residues 49–169 of GIRK1. Two major bands of ~4.5 and 6.0 kb RNA that hybridized to the probe are indicated with arrows. The positions of RNA size markers (9.5, 7.5, 4.4, 2.4, 1.4 and 0.2 kb) are shown on the right of each blot. The integrity of RNA samples were verified by ethidium bromide staining of the gel and by reprobing the same blot with a labelled cDNA for α -tubulin (1.6 kb message, data not shown). These controls, however, do not rule out the possibility that the smear in the rat atrium lane (*a*) might be due to partial degradation of RNA; much less smear was evident in the guinea-pig atrium lane (*b*).

METHODS. Northern analysis: poly(A)⁺ RNA was isolated using a Fast Track RNA isolation kit (Invitrogen) from tissues of 12-week-old rats (*a*) and 6-week-old guinea-pigs (*b*). Concentrations of the poly(A)⁺ RNA sample were determined by absorbance at 260 nm. Poly(A)⁺ RNA (3 μ g) was fractionated on 0.7% agarose-formaldehyde gel and transferred to a nylon membrane. Probes were labelled with ³²P by random priming. Hybridizations were as previously described¹².

The GIRK1 cDNA, a new member of the inwardly rectifying K⁺ channel superfamily, probably encodes the muscarinic K⁺ channel in the heart. Like the muscarinic K⁺ channel, the GIRK1 channel is abundant in the atrium and is likely to be activated directly by G proteins. Moreover, the single-channel conductance, kinetics and inward rectification properties are all similar to those of the muscarinic K⁺ channel. Mutagenesis and biochemical studies of GIRK1 and G-protein subunit(s) should provide insight into the interaction between G-protein subunit(s) and ion channels. □

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1. Trautwein, W. & Dudel, J. *Pflügers Arch.* **266**, 324–334 (1958).
2. Noma, A., Peper, K. & Trautwein, W. *Pflügers Arch.* **381**, 255–262 (1979).
3. Sakmann, B., Noma, A. & Trautwein, W. *Nature* **303**, 250–253 (1983).
4. Soejima, M. & Noma, A. *Pflügers Arch.* **400**, 424–431 (1984).
5. Breitwieser, G. E. & Szabo, G. *Nature* **317**, 538–540 (1985).
6. Logothetis, D. E., Kurachi, Y., Galper, J., Neer, E. J. & Clapham, D. E. *Nature* **325**, 321–326 (1987).
7. Yatani, A., Codina, J., Brown, A. M. & Birnbaumer, L. *Science* **235**, 207–211 (1987).
8. Yatani, A. et al. *Nature* **336**, 680–682 (1988).
9. Brown, A. M. & Birnbaumer, L. *A. Rev. Physiol.* **52**, 197–213 (1990).
10. Kurachi, Y., Tung, R. T., Ito, H. & Nakajima, T. *Prog. Neurobiol.* **39**, 229–246 (1992).
11. Ho, K. et al. *Nature* **362**, 31–38 (1993).
12. Kubo, Y., Baldwin, T. J., Jan, Y. N. & Jan, L. Y. *Nature* **362**, 127–133 (1993).
13. Bonner, T. I., Buckley, N. J., Young, A. C. & Brann, M. R. *Science* **237**, 527–532 (1987).
14. Gilman, A. G. *Cell* **36**, 577–579 (1984).
15. Dascal, N. *Crit. Rev. Biochem.* **22**, 317–387 (1987).
16. Jones, D. T. & Reed, R. J. *J. Biol. Chem.* **262**, 14241–14249 (1987).
17. Fong, H. K. W. et al. *Proc. natn. Acad. Sci. U.S.A.* **83**, 2162–2166 (1986).
18. Gautam, N., Baetscher, M., Aebersold, R. & Simon, M. I. *Science* **244**, 971–974 (1989).
19. Ito, H. et al. *J. gen. Physiol.* **98**, 517–533 (1991).
20. Morie, M. & Irisawa, H. *J. Physiol.* **408**, 313–332 (1989).

21. Ashcroft, S. J. H. & Achcroft, F. M. *Cell. Signal.* **2**, 197–214 (1990).
22. Vandongen, A. M. J. et al. *Science* **242**, 1433–1437 (1988).
23. Brown, D. A. *Rev. Physiol.* **52**, 215–242 (1990).
24. Stanfield, P. R., Nakajima, Y. & Yamaguchi, K. *Nature* **315**, 498–501 (1985).
25. Gahwiler, B. H. & Brown, D. A. *Proc. natn. Acad. Sci. U.S.A.* **82**, 1558–1562 (1985).
26. Williams, J. T., Colmers, W. F. & Pan, Z. Z. *J. Neurosci.* **8**, 3499–3506 (1988).
27. Mihara, S., North, R. A. & Surprenant, A. *J. Physiol.* **390**, 335–355 (1987).
28. Inoue, M., Nakajima, S. & Nakajima, Y. *J. Physiol.* **407**, 177–198 (1988).
29. North, R. A., Williams, J. T., Surprenant, A. & Christie, M. J. *Proc. natn. Acad. Sci. U.S.A.* **84**, 5487–5491 (1987).
30. Wimpey, T. L. & Chavkin, C. *Neuron* **6**, 281–289 (1991).
31. Gerber, U., Stevens, D. R., McCarty, R. W. & Greene, R. W. *J. Neurosci.* **11**, 3861–3867 (1991).
32. Frohman, M. A. *PCR Protocols* 28–38 (Academic, San Diego, 1990).
33. Baldwin, T. J. et al. *Neuron* **7**, 471–483 (1991).
34. Ito, H. et al. *J. gen. Physiol.* **99**, 961–983 (1992).
35. Yang, X.-C. & Sachs, F. *Science* **243**, 1068–1071 (1989).

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Lethal effect of the anti-Fas antibody in mice

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DURING mammalian development, many cells are programmed to die^{1,2} most mediated by apoptosis³. The Fas antigen⁴ coded by the structural gene for mouse lymphoproliferation mutation (*lpr*)^{5,6}, is a cell surface protein belonging to the tumour necrosis factor/nerve growth factor receptor family^{7,8}, and mediates apoptosis⁷. The Fas antigen messenger RNA is expressed in the thymus, liver, heart, lung and ovary⁸. We prepared a monoclonal antibody against mouse Fas antigen, which immunoprecipitated the antigen (M_r 45K) and had cytolytic activity against cell lines expressing mouse Fas antigen. We report here that staining of mouse thymocytes with the antibody indicated that thymocytes from the wild-type and *lpr*^{CG} mice expressed the Fas antigen, whereas little expression of the Fas antigen was found in *lpr* mice. Intraperitoneal administration of the anti-Fas antibody into mice rapidly killed the wild-type mice but neither *lpr* nor *lpr*^{CG} mice. Biochemical, histological and electron microscope analyses indicated severe damage of the liver by apoptosis. These findings suggest that the Fas antigen is important in programmed cell death in the liver, and may be involved in fulminant hepatitis in some cases.

Armenian hamsters were immunized with W4 cells, in which mouse WR19L cells had been transformed with mouse Fas antigen complementary DNA⁸. Hybridomas produced by fusing the spleen cells with mouse myeloma were screened by FACS for antibodies that bind to a human Jurkat cell transformant (J6) expressing mouse Fas antigen, but not to the parental Jurkat

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TABLE 1 Biochemical analysis of the serum of mice which received anti-Fas antibody

Time (h)	GOT (IU l ⁻¹)	GPT (IU l ⁻¹)	ALP (IU l ⁻¹)	AMY (S-U)	CPK (IU l ⁻¹)	HBD (IU l ⁻¹)	BUN (mg dl ⁻¹)	T-BIL (mg dl ⁻¹)	Na ⁺ (mEq l ⁻¹)	K ⁺ (mEq l ⁻¹)	Cl ⁻ (mEq l ⁻¹)	Neutrophils (× 10 ² m m ⁻³)	Lymphocytes (× 10 ² m m ⁻³)
0	102	30	582	2,610	2,688	561	26.1	0.06	147.9	5.61	116.2	16.7	60.8
1.0	87	30	525	2,727	1,656	468	25.2	0.03	148.4	5.62	116.0	11.6	47.6
2.0	1,320	1,274	480	3,080	6,340	1,268	25.4	0.18	142.2	7.74	114.3	19.6	28.9
3.0	21,360	31,545	759	2,013	6,180	26,460	30.9	0.42	139.9	8.54	113.4	8.6	34.6

The purified anti-Fas monoclonal antibody (Jo2) (100 µg) in 200 µl of PBS was injected intraperitoneally into 3-week-old female BALB/c mice. At indicated times after injection, blood samples were collected, and the various biochemical parameters of the serum were quantified using a standard clinical automatic analyser (Hitachi, Type 7150). The parameters examined were: GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; ALP, alkaline phosphatase; AMY, amylase; CPK, creatine phosphokinase; HBD, α -hydroxy-butyric dehydrogenase; BUN, blood urea nitrogen; T-BIL, total bilirubin. The number of white blood cells were counted using a haematocytometer, and the blood smear was stained with May-Giemsa solution for differential counts.

cell. One hybridoma (Jo2) was identified, and the antibody was purified on protein A-Sepharose. The antibody was pure (Fig. 1a), and seemed to be an IgG class because it bound to the protein A-Sepharose and had a M_r of about 160K on gel filtration. The Jo2 antibody specifically recognized the Fas antigen, as immunoprecipitation of the cell lysates from biotin-labelled W4 and WR19L cells⁹ with the antibody gave a 45K band in W4 cells but not in WR19L cells (Fig. 1b). The difference between the observed M_r and that deduced from its amino-acid sequence (M_r 34, 971) may be due to glycosylation. Previously, the Fas antigen mRNA was detected in the thymus⁸. Accordingly, most thymocytes from the wild-type mice (BALB/c and MRL +/+) were positive for the Fas antigen (Fig. 1c). There are two mouse mutants of the Fas antigen, *lpr* and *lpr^{cg}* (ref. 5). As expected from little expression of the Fas antigen mRNA in *lpr*-mice^{5,6}, Fas antigen was not detected on the cell surface of thymocytes from MRL-*lpr/lpr* mice (Fig. 1c). The *lpr^{cg}* mice express the Fas antigen mRNA which codes for the protein mutated in the cytoplasmic region⁵. Accordingly, the thymocytes from MRL-*lpr^{cg}/lpr^{cg}* mice¹⁰ were stained with the Jo2 antibody (Fig. 1c).

Monoclonal antibodies recognizing the human Fas antigen, anti-Fas or anti-APO-1 antibodies, work as agonists and induce apoptosis^{4,7,11,12}. The Jo2 antibody showed cytolytic activity against cells expressing the mouse Fas antigen. As shown in Fig. 2a, W4 cells, but not the parental WR19L cells, were killed by the Jo2 antibody in a dose-dependent manner. The Jo2 antibody was intraperitoneally injected into BALB/c mice. Most of the mice (14 out of 15) receiving 100 µg of the antibody were dead within 6 hours (Fig. 2b) and 10 µg of the antibody was effective in killing half of the mice (5 out of 10) within 8 hours. Administration of 100 µg of control hamster IgG had no effect on mouse viability. The endotoxin content of the Jo2 antibody was estimated to be 240 pg per 100 µg protein, which is far less than the lethal toxicity level for mice^{13,14}. C3H/HeJ mice, which are highly resistant to the lethal effect of endotoxin¹⁵, were also killed by the Jo2 antibody (Fig. 2b), confirming that the lethal effect of anti-Fas antibody is not due to endotoxin. When MRL +/+, MRL-*lpr/lpr* and MRL-*lpr^{cg}/lpr^{cg}* mice were injected with the Jo2 antibody, MRL-*lpr/lpr* and MRL-*lpr^{cg}/lpr^{cg}* were resistant to the antibody (Fig. 2b), whereas MRL +/+ mice were killed by the antibody as effectively as

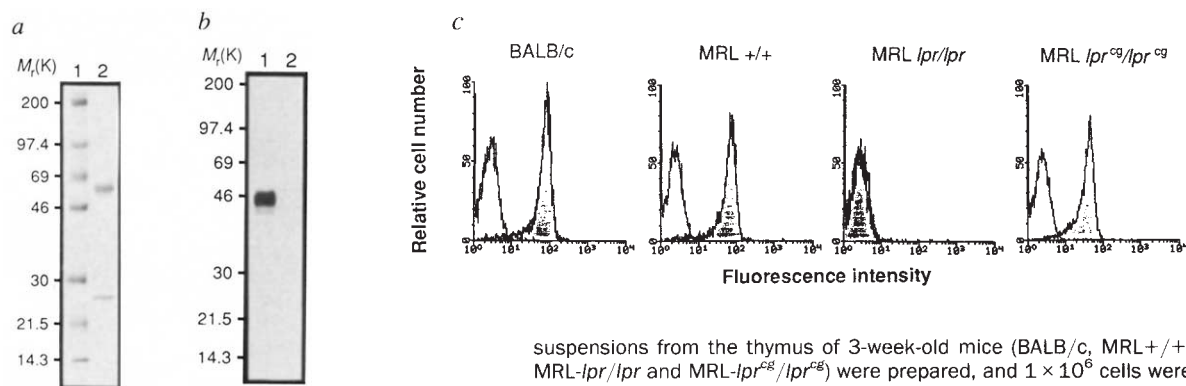


FIG. 1 a, SDS-polyacrylamide gel electrophoresis of the purified Jo2 antibody. The purified Jo2 antibody (2 µg; lane 2), and Rainbow Markers (Amersham) (lane 1) were mixed with SDS sample buffer containing 5% 2-mercaptoethanol. After heating at 95 °C for 3 min, samples were electrophoresed on a 10–20% gradient polyacrylamide gel in the presence of SDS, and proteins were stained by Coomassie brilliant blue. M_r marker proteins are shown on the left. b, Immunoprecipitation of mouse Fas antigen with the Jo2 antibody. W4 (lane 1) or WR19L cells (lane 2) were biotinylated, lysed in an NP-40-containing buffer, preadsorbed with normal hamster IgG and immunoprecipitated with the anti-Jo2 antibody, essentially as described⁹. The immunoprecipitates were heated at 95 °C for 3 min in SDS-sample buffer with 5% 2-mercaptoethanol, and electrophoresed through 10–20% gradient polyacrylamide in the presence of SDS. The proteins were blotted onto a PVDF membrane (Millipore) and detected using the ECL (enhanced chemiluminescence) system (Amersham) after staining with streptavidin conjugated horseradish peroxidase. c, Flow cytometric analysis of mouse thymocytes. Single-cell

suspensions from the thymus of 3-week-old mice (BALB/c, MRL +/+, MRL-*lpr/lpr* and MRL-*lpr^{cg}/lpr^{cg}*) were prepared, and 1×10^6 cells were stained with Jo2 antibody followed by phycoerythrin (PE)-conjugated goat anti-hamster IgG (F(ab')₂ fraction, CALTAG) (shaded area). The open area indicates the FACS profile of thymocytes stained with the second antibody alone.

METHODS. A WR19L cell transformant (W4) and a Jurkat cell transformant (J6) highly expressing mouse Fas antigen were established by introducing the mouse Fas antigen cDNA expression vector into the parental cells. Eight-week-old female Armenian hamsters (Nippon Crea) were intraperitoneally immunized with the 1×10^7 W4 cells (irradiated with 2,800 rads) 6 times at weekly intervals. Mouse myeloma P3X63Ag8U.1 cells were fused with spleen cells from the immunized hamster and hybridomas were selected in HAT medium as described^{25,26}. Hybridoma supernatants were examined for binding activity to mouse Fas antigen on J6 cells by a FACS analysis. One hybridoma cell line (Jo2) which gave a positive result was subcloned by limiting dilution, and grown in serum-free medium (AIM-V medium, Gibco). The antibody secreted from the Jo2 hybridoma was concentrated by precipitating with 50% ammonium sulphate, and purified by using protein A-Sepharose (Pharmacia) as described²⁷.

BALB/c mice (data not shown). As MRL-*lpr*^{cg}/*lpr*^{cg} mice express the Fas antigen which can interact with the Jo2 antibody (Fig. 1c), these findings indicate that the lethal effect of the anti-Fas antibody was due to the functional Fas antigen, and not complement-mediated cell lysis.

Biochemical analysis of the mouse serum showed that the amount of GOT, GPT, HBD and T-BIL in the serum dramatically increased 3 h after injection of the antibody, up to 200, 1,000, 50 and 10 times more than the basal level, respectively (Table 1). A marginal increase of ALP, AMY and BUN was observed, but there were no significant changes in the other parameters, such as differential blood cell counts and concentrations of Na⁺ and Cl⁻ ions, although K⁺ did increase slightly. (Table 1). These findings suggest that the Fas antibody damaged the liver. When tissue sections of mice were histologically examined 2 h after injection of the antibody, the liver, but not any other tissues, showed many areas of focal haemorrhage and necrosis (Fig. 3a). Only a few normal hepatocytes remained and most damaged cells were characterized by condensation of the cytoplasm, and pyknosis of nuclei. When the liver and heart sections were examined under the transmission electron micro-

scope (TEM), the liver sections showed many cells containing condensed and fragmented nuclei (Fig. 3b), which is characteristic of apoptosis³. These results indicate that individual hepatocytes died by an apoptotic process. Apoptotic cells are usually phagocytosed by neighbouring macrophages and granulocytes^{1,3}. In the Fas antibody-induced liver injury, the dead cells did not seem to be phagocytosed and the cell contents leaked out. This may be because the rate of cell death was so much greater than the rate of phagocytosis. The apparent normality of the thymus and heart expressing the Fas antigen⁸ may be because the mice died before any effects appeared in these tissues.

Administration of endotoxin, tumour necrosis factor (TNF) or interleukin-1 (IL-1) causes septic shock accompanied by leukocytosis, and eventually kills the animal by organ failure¹³⁻¹⁷. Administration of the anti-Fas antibody quickly killed the mice without significant changes in the blood cell counts, suggesting little involvement of inflammatory mediators in this process. From these findings, and the resistance of *lpr*⁻ and *lpr*^{cg} mice against the lethal effect of the Fas antibody, it is likely that the Fas antibody directly induced the death of hepatocytes expressing the Fas antigen (ref. 8 and unpublished results). The

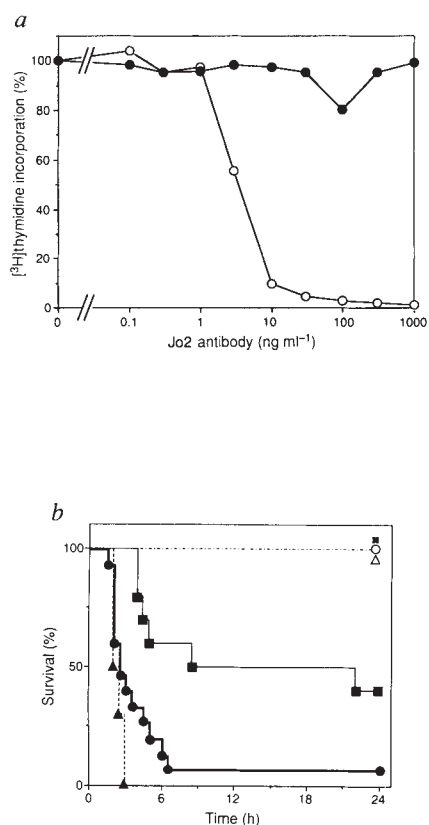


FIG. 2 a, Cytolytic activity of the Jo2 anti-mouse Fas monoclonal antibody. W4 cells (2×10^4) (○) or WR19L cells (●) were mixed with various concentrations ($0.1-1,000 \text{ ng ml}^{-1}$) of the Jo2 antibody in a 96-well microtitre plate. After incubation at 37°C for 16 h, $0.5 \mu\text{Ci}$ [^3H]thymidine (specific activity, 74 GBq mmol^{-1}) was added per well and incubated for a further 4 h before collection. The results are presented as percentage of the [^3H]thymidine incorporation observed without the Jo2 antibody. b, Lethal effect of anti-Fas antibody in mice. $100 \mu\text{g}$ of the purified Jo2 antibody was intraperitoneally injected into 3-5-week-old female mice of BALB/c (●), C3H/HeJ (▲), MRL-*lpr*/*lpr* (○) and MRL-*lpr*^{cg}/*lpr*^{cg} (△). BALB/c mice also received $10 \mu\text{g}$ of the Jo2 antibody (■) or $100 \mu\text{g}$ of the normal hamster IgG (×). The number of mice in each group was 7-15, and percentages of mice surviving until the indicated time were plotted.

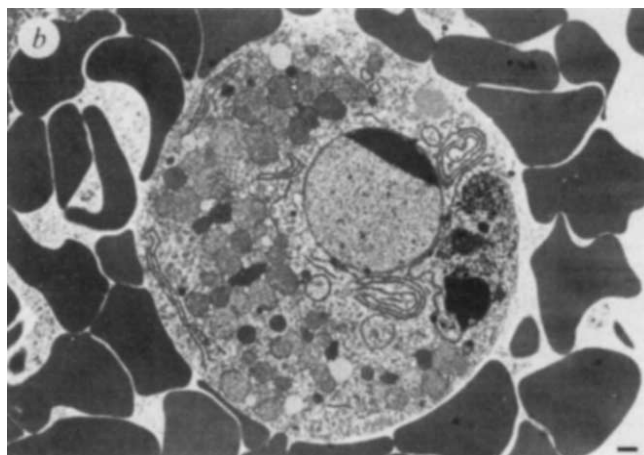
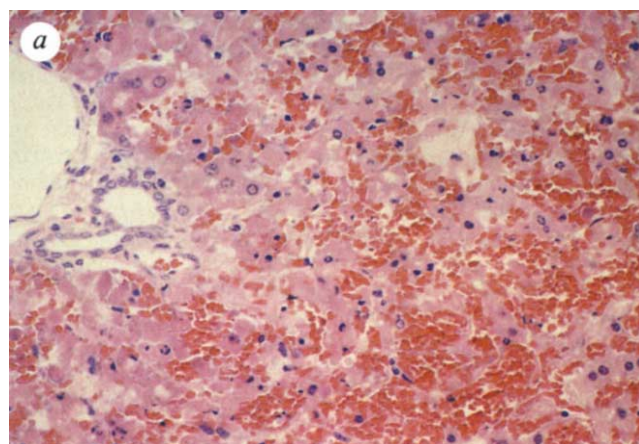


FIG. 3 The anti-Fas antibody induced apoptosis in the liver. Three-week-old BALB/c mice were injected with $100 \mu\text{g}$ of the Jo2 antibody. Liver sections were prepared 2 h after injection. a, Histological analysis of the liver section after staining with haematoxylin and eosin, $\times 20$. Most hepatocytes carry pyknotic nuclei. Many red blood cells penetrate the liver. b, TEM of an apoptotic cell containing condensed and fragmented nuclei. Many red blood cells surround the apoptotic cell. Bar, $1 \mu\text{m}$.

liver showed gross injury by administration of the anti-Fas antibody and acute hepatic failure seems to be responsible for the in tissues such as the heart and lung. Why do the liver cells express the Fas antigen which mediates apoptosis? In normal rat liver, apoptotic bodies were observed in the acinar zone 3, where most senescent cells accumulate¹⁸. Administration of the anti-Fas antibody induced the death of hepatocytes in the entire region of the liver. It is possible that the natural ligand for the Fas antigen is only expressed in a restricted area and controls the death of hepatocytes, so maintaining homeostasis in the liver. Another possible explanation is that the Fas antigen mediates apoptosis of cells carrying DNA damaged by carcinogens or chemical drugs introduced into the liver¹⁹. In any event, the hepatic failure observed by the anti-Fas antibody administration would be a good model system for human fulminant hepatitis, which can be caused by activation of the immune system such as cytotoxic T-cells²⁰. It is therefore noteworthy that a Fas ligand has been found on the cell surface of a cytotoxic T-cell line²¹. It is possible that abnormally activated Fas ligand and Fas antigen systems play a role in fulminant hepatitis. Recently, possible therapeutic uses of the agonistic antibodies against the Fas antigen were proposed for the treatment of EBV-induced lymphoproliferative lesions in immunocompromised individuals²², HTLV-1 associated malignant disorders²³ or AIDS patients²⁴. Such approaches must be carefully controlled in the light of our present results. □

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1. Raff, M. C. *Nature* **356**, 397–400 (1992).
2. Ellis, R. E., Yuan J. & Horvitz, H. R. *Rev. Cell Biol.* **7**, 663–698 (1991).
3. Wyllie, A. H., Kerr, J. F. R. & Currie, A. R. *Int. Rev. Cytol.* **68**, 251–306 (1980).
4. Yonehara, S., Ishii, A. & Yonehara, M. *J. exp. Med.* **169**, 1747–1756 (1989).
5. Watanabe-Fukunaga, R., Brannan, C. I., Copeland, N. G., Jenkins, N. A. & Nagata, S. *Nature* **356**, 314–317 (1992).
6. Adachi, M., Watanabe-Fukunaga, R. & Nagata, S. *Proc. natn. Acad. Sci. U.S.A.* **90**, 1756–1760 (1993).
7. Itoh, N. *et al. Cell* **66**, 233–243 (1991).
8. Watanabe-Fukunaga, R. *et al. J. Immun.* **148**, 1274–1279 (1992).
9. Meier, T., Arni, S., Malarkannan, S., Poincelot, M. & Hoessli, D. *Analyt. Biochem.* **204**, 220–226 (1992).
10. Kimura, M. *et al. Immunology* **76**, 498–504 (1992).
11. Trauth, B. C. *et al. Science* **245**, 301–305 (1989).
12. Oehm, A. *et al. J. Biol. Chem.* **267**, 10709–10715 (1992).
13. Lehmann, V., Freudenberg, M. A. & Galanos, C. *J. exp. Med.* **165**, 657–663 (1993).
14. Eskandari, M. K. *et al. J. Immun.* **148**, 2724–2730 (1992).
15. Watson, J., Kelly, K., Largen, M. & Taylor, B. A. *J. Immun.* **120**, 422–424 (1978).
16. Waage, A. & Espevik, T. *J. exp. Med.* **167**, 1987–1992 (1988).
17. Tracey, K. J. *et al. Science* **234**, 470–474 (1986).
18. Benedetti, A., Jezaquel, A. M. & Orlandi, F. *J. Hepat.* **7**, 319–324 (1988).
19. Potten, C. S. *Cancer Metastasis Rev.* **11**, 95–103 (1992).
20. Chisari, F. V. *Molec. Gen. Med.* **2**, 67–103 (1992).
21. Rouvier, E., Luciani, M.-F. & Golstein, P. *J. exp. Med.* **177**, 195–200 (1993).
22. Falk, M. H. *Blood* **79**, 3300–3306 (1992).
23. Debatin, K.-M., Goldmann, C. K., Bamford, R., Waldman, T. A. & Krammer, P. H. *Lancet* **335**, 497–500 (1990).
24. Kobayashi, N. *et al. Proc. natn. Acad. Sci. U.S.A.* **87**, 9620–9624 (1990).
25. Sanchez-Madrid, F., Szklut, P. & Springer, T. A. *J. Immun.* **130**, 309–312 (1983).
26. Hirata, Y. *et al. J. Immun.* **143**, 2900–2906 (1989).
27. Cligan, J. E., Krusbeek, A. M., Hargulies, D. H., Shevach, E. M. & Strober, W. *Current Protocols in Immunology* (Wiley Interscience, New York, 1991).

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Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx

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INTRACELLULAR Ca^{2+} signals that last more than a few minutes after the onset of stimulation depend critically on influx of extracellular Ca^{2+} . Such Ca^{2+} influx can be triggered in many cell types by depletion of intracellular Ca^{2+} stores without detectable elevations of known messengers. The mechanism by which store depletion can control plasma membrane Ca^{2+} permeability remains controversial^{1–7}. Here we present evidence for a novel soluble mediator. Calcium depletion of a lymphocyte cell line caused the messenger to be released from intracellular organelles into the cytoplasm and to a much lesser extent into the extracellular medium. The messenger caused Ca^{2+} influx when applied to macrophages, astrocytoma cells, and fibroblasts and was therefore named CIF (for Ca^{2+} -influx factor). CIF appears to have hydroxyls (or hydroxyl and amino groups) on adjacent carbons, a phosphate, and a M_r under 500.

We chose the Jurkat line of human tumour lymphocytes as producers of the putative messenger because they display Ca^{2+} influx that can be triggered by emptying of internal stores^{8–10} and are easily grown in large quantity at high density. Concentrated suspensions of Jurkats were stimulated for 2 min with phytohaemagglutinin (PHA) in zero Ca^{2+} medium, lysed at pH 1 to stop synthesis and degradation of messengers, and centrifuged to remove cell debris. The lymphocyte extracts were neutralized

to physiological pH and tested extracellularly on P388D1 macrophage-related cells, 1321N1 astrocytoma cells, and REF-52 fibroblasts. These three cell lines were physiologically unrelated and insensitive to PHA. To avoid effects of ATP or fatty acids, which increase internal Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in various cell types^{11,12}, the cell extract was treated with hexokinase, glucose, and fatty acid-free bovine serum albumin (BSA, 20 mg ml⁻¹). Control experiments verified that these additions prevented exogenous ATP and fatty acids from having any effect. Typical $[\text{Ca}^{2+}]_i$ responses are shown in Fig. 1. Addition of Jurkat extract triggered a sustained but fluctuating $[\text{Ca}^{2+}]_i$ increase in all three cell types. When the extract was further diluted, the amplitude of the response decreased and the latency increased. Such experiments showed that the P388D1 macrophages were the most sensitive detectors and the REF-52 fibroblasts were the least. Jurkat cells were not the only cells that produced the messenger, because analogous extracts prepared from P388D1 macrophages stimulated with platelet-activating factor caused $[\text{Ca}^{2+}]_i$ elevations in astrocytoma cells similar to those shown in Fig. 1b, yet astrocytoma cells did not respond directly to platelet-activating factor (data not shown).

Further analysis of the $[\text{Ca}^{2+}]_i$ increase induced in astrocytoma cells is presented in Fig. 2. In the presence of external Mn^{2+} (Fig. 2a), Jurkat extract not only elevated $[\text{Ca}^{2+}]_i$ but simultaneously accelerated Mn^{2+} quenching of the fura-2, just as expected in non-excitable cells for Ca^{2+} entry pathways because they usually also conduct Mn^{2+} (refs 6, 8, 12, 13). In Ca^{2+} -free medium (Fig. 2b), the Jurkat extract had no effect on $[\text{Ca}^{2+}]_i$, but readdition of extracellular Ca^{2+} restored the normal $[\text{Ca}^{2+}]_i$ elevation. Econazole, a blocker of stimulated Ca^{2+} and Mn^{2+} entry in various cell types^{6,14}, considerably inhibited the effect of cell extract. Finally, the content of the intracellular Ca^{2+} pools after stimulation with cell extract was compared with results from a blank (Fig. 2c) or the muscarinic agonist carbachol (Fig. 2d). Jurkat extract caused no lasting loss of stored Ca^{2+} , as revealed by the large $[\text{Ca}^{2+}]_i$ transient when those stores were finally dumped by Ca^{2+} ionophore in EGTA medium. By contrast, muscarinic stimulation immediately dumped the internal Ca^{2+} stores. Although the extract and carbachol gave roughly matching levels of Ca^{2+} entry while external Ca^{2+} was present, carbachol kept the stores mostly depleted as

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shown by the final small response to ionomycin in EGTA. These results together confirm that the Jurkat extract applied to astrocytoma cells stimulated Ca^{2+} influx directly rather than as a secondary consequence of triggered release from the stores of those responder cells. The same appeared to hold for REF-52 fibroblasts though they were studied in less detail. But the response of the P388D1 macrophages was more complex and included some transient release of Ca^{2+} from internal stores as well as sustained Ca^{2+} influx.

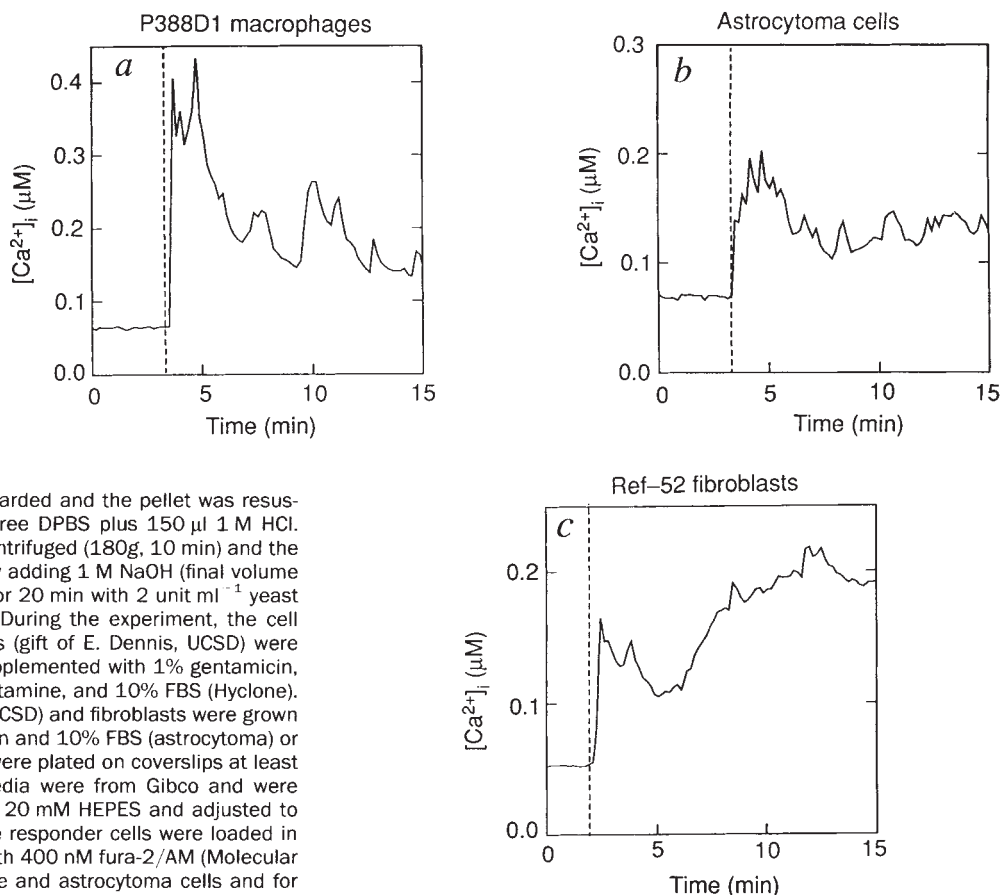
We then examined how the amount and subcellular distribution of the messenger were affected by stimulation of the producer Jurkat cells. Half of a large batch of Jurkats was stimulated with PHA in zero Ca^{2+} for 10 min, and the other half was left unstimulated in normal medium. Each suspension was then separated into supernatant and cells. The latter were subdivided into cytosolic and organellar fractions using digitonin to permeabilize the plasma membranes selectively. After parallel workups to complete lysis, inactivate enzymes, and remove digitonin, ATP, and fatty acids, the fractions were tested on macrophages as detector cells (Fig. 3a). The cell supernatant contained a small but definite amount of Ca^{2+} -elevating activity, which increased by 1.9-fold upon PHA stimulation. PHA stimulation caused much greater (sixfold) enhancement of activity in the cytosol and an almost equal loss of activity from the organelles. The total activity obtained by adding the activities of the three fractions was conserved upon stimulation.

Does release of activity depend on stimulation of surface receptors, elevation of cytosolic Ca^{2+} , or depletion of internal Ca^{2+} stores? Stimulatable release of activity into the cytosol persisted after loading the lymphocytes with an intracellular buffer to suppress even a transient increase in $[\text{Ca}^{2+}]_i$ and could be detected by astrocytoma cells (Fig. 3b). Another comparison (Fig. 3c) was between PHA in the presence or absence of extracellular Ca^{2+} , thapsigargin to inhibit Ca^{2+} -sequestering pumps in the organelles, or simple removal of external Ca^{2+} for long enough to deplete those organelles without any drug treatment or elevation of $[\text{Ca}^{2+}]_i$. Activities were tested in the supernatants of intact cells rather than in digitonin lysates because much less sample handling and cleanup were required, only needing addition of hexokinase to eliminate ATP. PHA and thapsigargin in normal external Ca^{2+} both gave the same ~2.5-fold stimulation of messenger release. Incubation in zero Ca^{2+} was almost as effective by itself and did not further enhance the activity due to PHA. All four conditions of store depletion, using three different mechanisms, gave messenger releases statistically different from the control but not from each other.

Various known messengers were tested to see if they could explain the activity and induce similar $[\text{Ca}^{2+}]_i$ rises in macrophages or astrocytoma cells. If $[\text{Ca}^{2+}]_i$ increases were observed, cross-desensitization experiments were tried, in which macrophages or astrocytoma cells were stimulated several times with the candidate until they became desensitized, after which the

FIG. 1 Increases in cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_i$) in P388D1 macrophages (a), 1321N1 astrocytoma cells (b) and REF-52 fibroblasts (c), in response to an extract of stimulated Jurkat lymphocytes.

METHODS. Jurkat T cells were grown in suspension in RPMI 1640, 1% penicillin/streptomycin and 5% heat-inactivated fetal bovine serum (FBS, Gibco). A suspension (50 ml), containing 0.3–0.4 ml packed cell volume, was centrifuged (180g, 4 min) and resuspended in 15 ml of Ca^{2+} -free Dulbecco's phosphate buffered saline (DPBS) with 0.5 mM EGTA. Phytohaemagglutinin (PHA, $20 \mu\text{g ml}^{-1}$) was added. After 2 min stimulation, the cells were centrifuged. The supernatant was discarded and the pellet was resuspended in 700 μl of nominally Ca^{2+} -free DPBS plus 150 μl 1 M HCl. After 20 min, the suspension was recentrifuged (180g, 10 min) and the supernatant brought back to pH 7.3 by adding 1 M NaOH (final volume 1 ml). The cell extract was then kept for 20 min with 2 unit ml^{-1} yeast hexokinase (Calbiochem) and frozen. During the experiment, the cell extract was kept on ice. P388D1 cells (gift of E. Dennis, UCSD) were cultured in Iscove's DMEM (IMDM) supplemented with 1% gentamicin, 5% non-essential amino acids, 2% glutamine, and 10% FBS (Hyclone). Astrocytoma cells (gift of J. H. Brown, UCSD) and fibroblasts were grown in MEM plus 1% penicillin/streptomycin and 10% FBS (astrocytoma) or 10% calf serum (fibroblasts). All cells were plated on coverslips at least 1 day before the experiments. All media were from Gibco and were supplemented by 11 mM glucose and 20 mM HEPES and adjusted to pH 7.3. For $[\text{Ca}^{2+}]_i$ measurements, the responder cells were loaded in Hank's balanced salt solution (HBS) with 400 nM fura-2/AM (Molecular Probes) for 20–30 min for macrophage and astrocytoma cells and for 45 min for fibroblasts. $[\text{Ca}^{2+}]_i$ was measured at 5-s intervals at room temperature on a ratio imaging system previously described³⁰. The traces are average $[\text{Ca}^{2+}]_i$ values of 6–12 cells in the same field. Where indicated by the dashed lines, 200 μl Jurkat extract (derived from 60–80 μl packed cells) supplemented with 20 mg ml^{-1} fatty acid-free



BSA (Calbiochem) and 1.3 mM Ca^{2+} were added to the responder cells in 400 μl HBS, which also contained 1.3 mM Ca^{2+} . In c, the volumes of extract and HBS were reversed because REF-52 cells were less sensitive.

TABLE 1 Analysis of CIF

(a) Comparison of known messengers with the new activity				(b) Chemical characterization of CIF	
	[Ca ²⁺] _i rise in macrophages	[Ca ²⁺] _i rise in astrocytoma	Cross- desensitization		Activity (%)
ATP ¹¹ (100 µM)	+++	+	No	Control	100
ADP (100 µM)	++	+	No	After 45 min at room temperature	88
GTP (100 µM)	++	—	No	Heat inactivation 70 °C, 20 min	195*
Ado, Guo, cAMP, cGMP ⁷ , AMP, GMP, cADPR ²⁵ (100 µM)	—	—	—	Protease 5 mg ml ⁻¹ , 30 min	204*
InsP ₃ (50 µM) + InsP ₄ (50 µM)	—	—	No	pH 13, 20 min	156
LPA ²⁶ (50 µg/ml)	++	++	No	Cutoff filter (M _r ≤ 500)	184*
cis-unsaturated fatty acids ¹² (30 µM)	—	—	—	Charcoal column (flow-through)	0*
LTC ₄ ²⁷ (10 µM)	+++	—	No	C18 column	
LTB ₄ (10 µM)	—	—	—	Flow-through	18*
PAF (20 nM)	+++	—	No	20% MeOH	67
Sph(1)P ^{28,29} (50 µM)	—	+	No	100% MeOH	0*
				Flow-through of	
				Mixed bed column	0*
				Cation exchange column	86
				Anion exchange column	7*
				Anion exchange column at pH 2.5	0*
				Alkaline phosphatase, Calf intestine	
				1 unit ml ⁻¹ , 20 min	0*
				Periodate	
				5 mM, 20 min	89
				50 mM, 20 min	0*
				Iodoacetamide 50 mM, 2 h	114
				NaBH ₄ 50 mM, 30 min	126

a, For each compound, the maximal dose tested is indicated. When a candidate was able to induce [Ca²⁺]_i increases in macrophages and astrocytoma cells, cross-desensitization experiments were done where indicated. *Cis*-unsaturated fatty acids (all from Cayman Chemical) were arachidonic, docosahexaenoic, linoleic, γ -linoleic, and oleic acids. Sphingosine-1-phosphate, Sph(1)P, was a gift of T. Ghosh and D. Gill²⁸. Cyclic ADP-ribose (cADPR) was a gift of H. C. Lee²⁵. In addition to the lack of cross-desensitization with CIF, further evidence against CIF being Sph(1)P was obtained from tests on REF52 fibroblasts. Unlike CIF, Sph(1)P caused [Ca²⁺]_i increases in fibroblasts at much lower doses (~1 µM) than required for the astrocytoma cells (~50 µM), and the fibroblast responses were almost entirely due to release from internal Ca²⁺ stores, in agreement with previous reports²⁹. b, Lymphocyte supernatant was prepared using PHA stimulation in zero Ca²⁺, subjected to the indicated manipulations and finally assayed on macrophages as described in Fig. 3. The values correspond to the mean [Ca²⁺]_i increase induced in two to five dishes and have been normalized to appropriate controls as follows. For cutoff filters, C₁₈ reverse-phase, charcoal and ion exchange columns, the control was the activity of the supernatant before the filter or column. Methanolic fractions were evaporated to dryness before reconstitution in the same volume of HBS. For stability tests other than pH extremes, the control activity was measured right after supernatant isolation. For pH stability tests, the same amount of NaOH and HCl were premixed and added to the control to mimic the dilution. In all other cases, the supernatant was split into two fractions; one was treated as indicated and the other one, the control, was kept at room temperature at the same pH for the same amount of time. Iodoacetamide treatment was for 2 h at pH 8, followed by quenching with 60 mM glutathione for 20 min, then neutralization to pH 7.3 with HCl. Both treated and control fractions were then passed through a C₁₈ reverse-phase column, which retained the activity and allowed excess reagents to flow through. The fraction eluted by 20% MeOH was then analysed. Excess periodate was similarly removed by reverse phase columns. C₁₈ reverse phase columns were 3 cm long, packed with 40 µm prep LC packing (Bakerbond Octadecyl, J. T. Baker) and washed with 7–10 ml buffer. Excess borohydride was removed by two additions and evaporations of methanol. Cutoff filters were Centri/Por microconcentrators, MWCO 500, Spectrum. Mixed-bed, cation, and anion exchangers were AG501-X8(D), Dowex 50W-X8 (H⁺ form), and AG1-X8 (formate form), respectively (BioRad). Protease was type IX bacterial from *Bacillus polymyxa*, Sigma P-6141. Calf intestinal alkaline phosphatase was from Boehringer. The values statistically different from the control (Student's *t*-test, *P* < 0.05) are indicated by asterisks.

Jurkat supernatant or extract was applied. Table 1a summarizes results arguing that the supernatant activity could not be attributed to any of the candidates listed. Though some ATP was detectable in raw supernatants, activity survived hexokinase/glucose treatment as applied in all the previous traces. This remaining activity was unaffected by prior desensitization of the ATP response. ADP and GTP could be excluded because they activate the same receptors on macrophages as ATP¹⁵ and show cross-desensitization. Many other candidates were excluded by the above criteria. Also, the mediator was probably not an eicosanoid or other metabolite of arachidonic acid, because supernatant activity continued despite 15 min pretreatment of the Jurkat cells with the phospholipase A₂ inhibitor 4-bromophenacyl bromide (20 µM) or blockage of eicosanoid production with nordihydroguaiaretic acid (20 µM), 5,8,11,14-eicosatetraynoic acid (50 µM)⁶, or indomethacin (25 µM). Putative inhibitors of cytochrome P450^{6,16} such as econazole (20 µM) or carbon monoxide (saturated at 1 atm) also failed to prevent Jurkats from releasing activity into the supernatant.

The chemistry of the Ca²⁺-influx factor or CIF was then further characterized (Table 1b) starting from cell supernatant

because it should be cleaner than whole-cell extracts. CIF seemed stable and not protein-like, because heat or protease treatment or passage through a M_r 500 cutoff filter enhanced the activity. Such enhancement might be explained if the activity in crude supernatant had been weakened by partial binding to protein. But activity of heat-treated supernatant could not be reduced by BSA, so any such protein binding did not seem totally nonspecific. The small size of CIF was confirmed by size exclusion chromatography through Bio-Gel P-2 polyacrylamide. Even though the activity passed through the small short cartridges of reverse-phase silica used to filter out digitonin, CIF did seem to have some hydrophobic portions, because it was mostly retained on larger and longer columns of chromatographic-grade C₁₈ reverse-phase silica. Elution of such columns with buffer containing 20% methanol allowed most of the activity to be recovered, but no further activity could be eluted with 100% methanol. Charcoal columns also filtered out all the activity. Although these retentions suggested at least some hydrophobicity, extraction of supernatants with chloroform-methanol (1 : 1 v/v) left all the activity in the aqueous phase, even when the latter was acidified to pH 2.5 to protonate carboxylate groups. Cation

exchange columns let the activity through whereas anion exchange or mixed bed columns trapped it, suggesting that the messenger was negatively charged. Acidification to pH 2.5 did not prevent retention by the anion exchange column, suggesting again that the relevant charge was more likely to reside on a phosphate (pK_a typically 1–2) than a carboxylate (pK_a typically 4–5). The supernatant activity completely disappeared after treatment with alkaline phosphatase (Table 1b). The sensitivity to alkaline phosphatase and the extraction and ion-exchange behaviour argue for an essential phosphate in the molecule. Exposure to pH 13 for 20 min did not significantly affect the activity, suggesting the absence or unimportance of readily hydrolysable ester linkages. Periodate (50 mM), which cleaves vicinal diols or aminoalcohols to carbonyl compounds and oxidizes thiols or thioethers, completely destroyed the activity in 20 min. This effect of periodate was probably not due to thiol groups

because the activity was insensitive to iodoacetamide, which should alkylate thiols. Finally, insensitivity to borohydride suggested that aldehydes, ketones, or reducing sugars were absent or non-essential.

The main short-term effect of depletion of Ca^{2+} stores seems not to be fresh biosynthesis of the CIF but rather its movement from organelles into the digitonin-releasable fraction, the cytosol. The mechanism for such release from storage is unknown but unlikely to be through the inositol 1,4,5-trisphosphate ($InsP_3$)-activated Ca^{2+} channels, because the latter are believed to be cation-selective and are not activated by thapsigargin or by Ca^{2+} -depletion of the cytosol. Precedent exists for Ca^{2+} -dependent release of anions from the $InsP_3$ -sensitive stores. Recently, Fulceri *et al.*¹⁷ have shown that inorganic phosphate accumulates in microsomal vesicles when the latter are allowed to take up Ca^{2+} ; release of the microsomal Ca^{2+} by $InsP_3$, Ca^{2+} -

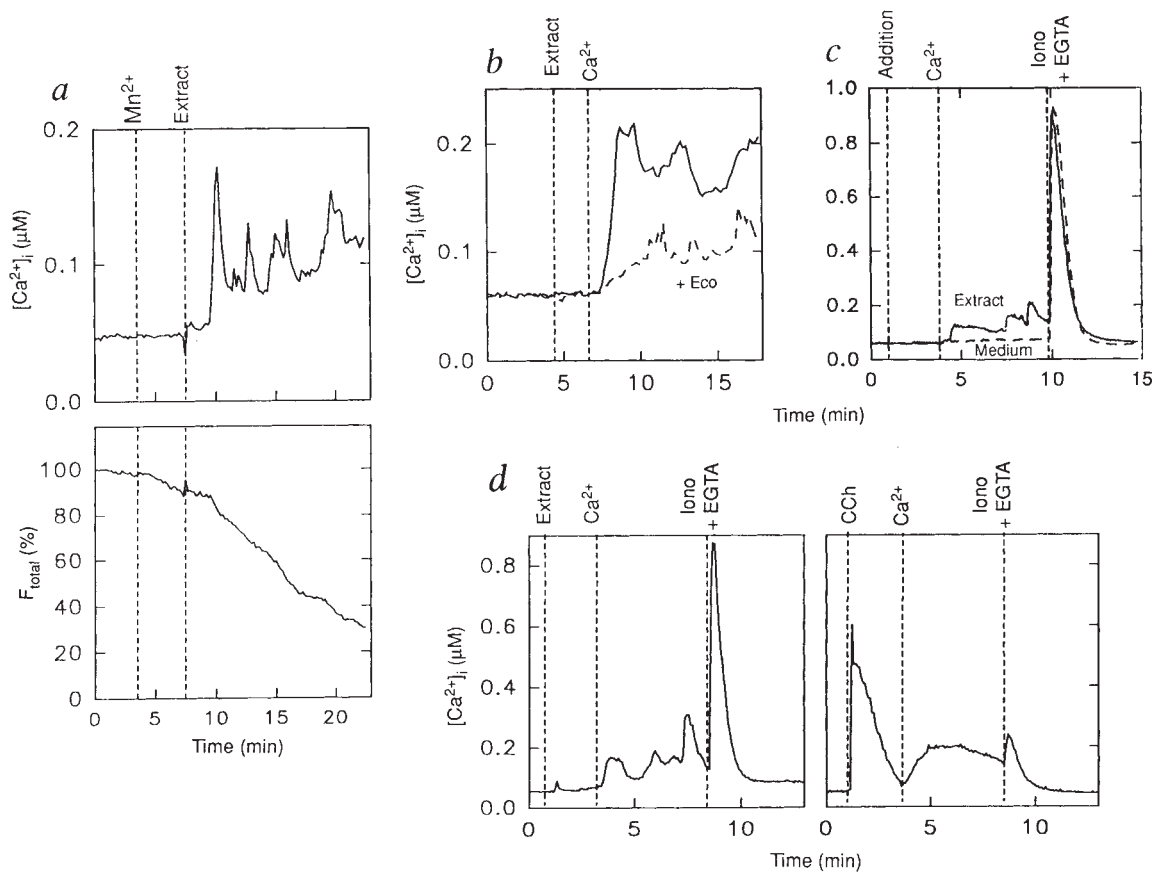


FIG. 2 Lymphocyte extract causes Ca^{2+} influx but not release of internal Ca^{2+} stores in astrocytoma cells. a, $[Ca^{2+}]_i$ (top) and total Ca^{2+} -independent fluorescence (F_{total} , bottom) were simultaneously measured in astrocytoma cells in 1.3 mM external Ca^{2+} after addition of 0.4 mM Mn^{2+} and lymphocyte extract. The latter induced a $[Ca^{2+}]_i$ increase in parallel with an acceleration of Mn^{2+} -quenching of F_{total} . b, In Ca^{2+} -free DPBS medium with 0.5 mM EGTA, lymphocyte extract did not increase $[Ca^{2+}]_i$ (solid line) until external Ca^{2+} (2 mM) was readmitted. Econazole (5 μM, applied 3 min before extract) blunted the $[Ca^{2+}]_i$ increase (dashed line) after external Ca^{2+} readmission. c, Lymphocyte extract (solid line) or control buffer (dashed line) was added to astrocytoma cells in DPBS with no added Ca^{2+} and 0.5 mM EGTA. Ca^{2+} (2 mM) was added, followed by 2 μM ionomycin and 10 mM EGTA. d, The astrocytoma cells were treated as in c with lymphocyte extract (left) or 100 μM carbachol (right). The sizeable fluctuations in average $[Ca^{2+}]_i$ in response to lymphocyte extract (Figs 1 and 2) reflect even larger fluctuations in single responder cells and may be due to occasional Ca^{2+} -induced Ca^{2+} -release from their fully loaded Ca^{2+} stores. b–d,

Show that any such release from internal stores is secondary to the Ca^{2+} influx. Analogous fluctuations (data not shown) are generated by a threshold dose of digitonin, another treatment that should likewise induce Ca^{2+} entry without directly discharging internal Ca^{2+} stores. By contrast, the Ca^{2+} influx generated by carbachol in astrocytoma cells (Fig. 2d) is smoother and less prone to amplification because the Ca^{2+} stores have already been depleted as a primary step.

METHODS. Lymphocyte extract was prepared and added to the astrocytoma cells as described in Fig. 1 except that 0.5 mM EGTA was included in the extract for (b–d) instead of 1.3 mM Ca^{2+} for a. Each trace shows the average $[Ca^{2+}]_i$ of 3–12 astrocytoma cells in the same dish. F_{total} was defined as (fluorescence at 350 nm excitation + 0.22 × fluorescence at 385 nm excitation); the coefficient 0.22 was determined in control experiments to simulate the isoexcitation wavelength and make F_{total} insensitive to $[Ca^{2+}]_i$ changes imposed by ionomycin, as described previously³¹. The F_{total} values have been normalized to 100% corresponding to the mean fluorescence during the first 2 min of recording.

ionophore, or chelation of external Ca^{2+} causes parallel release of the phosphate with no discernable additional time delay, a few seconds or less in the case of InsP_3 or ionophore. A similar Ca^{2+} -dependent release of CIF, which is a phosphate-containing anion, would be an economical mechanism for gating the signal to activate Ca^{2+} influx. But the precise timing of CIF release is unknown, so more complex mechanisms involving enzymatic steps cannot be excluded. Also, Ca^{2+} influx cannot depend entirely on prestored CIF, because depletion-induced Ca^{2+} permeability can be quite long lasting. Under those conditions, CIF may be continually produced in the organelles and released into the cytosol. In addition, the present results do not rule out other mechanisms^{3,7,18,22} that might trigger Ca^{2+} influx in parallel. CIF seems to have some ability to cross intact plasma membranes, because perhaps 10–20% of the cytosolic CIF appears in the supernatant of stores-depleted cells even without digitonin permeabilization. This fraction is larger than would be readily explained by the few per cent of leaky cells. CIF showed detect-

able activity when applied extracellularly to three unrelated cell types, macrophages, fibroblasts, and astrocytoma cells, showing that its action may have some generality. Present evidence does not show whether it acts from the outside or inside the target cells. A receptor on the intracellular face of the plasma membrane would make more sense for a signal released from organelles, but may seem difficult to reconcile with the observed effectiveness of an anionic, phosphate-containing messenger administered outside. But translocases might be present not only in the organellar membranes but also the plasma membranes both of generator and of responder cells. The modest absolute amplitude of the $[\text{Ca}^{2+}]_i$ increases in our responder cells may well reflect administration on the 'wrong' side of a permeability barrier, and the variations in sensitivity of different responder cell types might well reflect differences in permeability rather than receptor affinity. Direct intracellular delivery of CIF by whole-cell patch clamp should be informative. Nevertheless, because CIF shows detectable extracellular potency and might

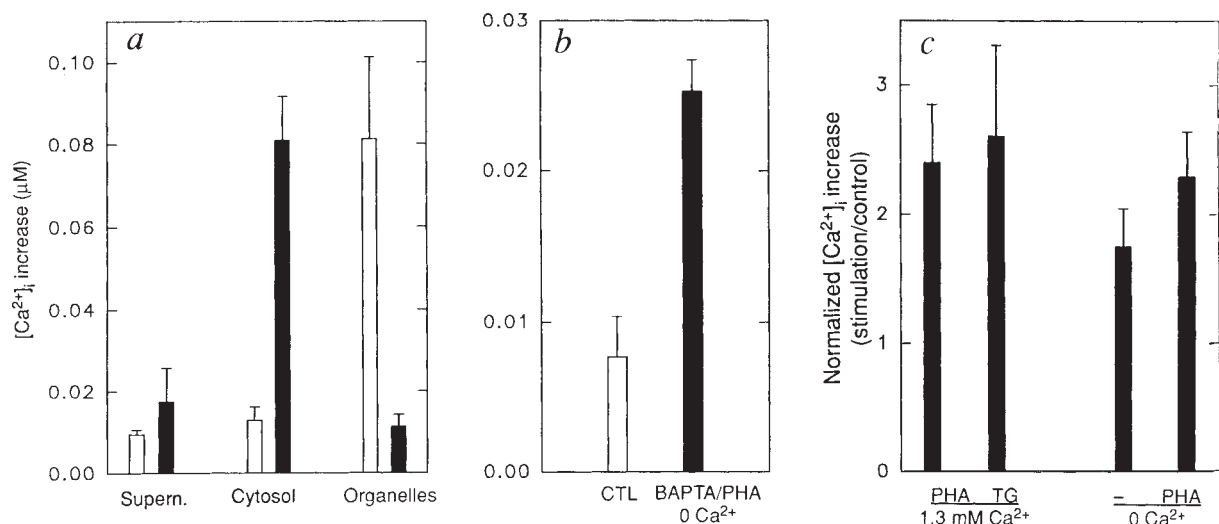


FIG. 3 Location of the messenger within Jurkat lymphocytes and ways of stimulating its release. *a*, $[\text{Ca}^{2+}]_i$ -elevating activities in the supernatant, the cytosol and the organelles of nonstimulated (open bars) or stimulated (20 μg ml⁻¹ PHA in Ca^{2+} -free DPBS plus 0.5 mM EGTA for 10 min, solid bars) lymphocytes were assayed on macrophages. Each suspension of lymphocytes (10% cytotrit) was fractionated by centrifugation, removal of the supernatant, and resuspension in a matching volume of nominally Ca^{2+} -free DPBS with 50 μM digitonin to permeabilize the plasma membranes selectively. Control experiments showed that 50 μM was the minimum dose of digitonin for complete permeabilization as assessed by trypan blue. This high dose probably reflects depletion of the digitonin by the high concentration of cells. The soluble cytosolic contents thus released were separated from intracellular organelles and insoluble residues by recentrifugation after 5 min; the pellet was resuspended in the same volume of nominally Ca^{2+} -free DPBS. All fractions were immediately acidified to pH 2.5, kept for 20 min to inactivate breakdown enzymes (and to extract the final pellet), filtered through two small cartridges of reverse-phase octadecyl silica (SPICE C₁₈, Rainin) to remove digitonin and fatty acids, neutralized to pH 7.3, treated with 2 units ml⁻¹ hexokinase for 20 min, and supplemented with 1.3 mM Ca^{2+} . $[\text{Ca}^{2+}]_i$ increases were determined on macrophages as the difference between the resting level of $[\text{Ca}^{2+}]_i$ and the maximal $[\text{Ca}^{2+}]_i$ value reached within 7 min after addition of extract (diluted 1:1 with HBS to a final concentration equivalent to the contents of 30–40 μl packed Jurkat cells in 600 μl medium). Each value corresponds to the average ± s.e. of responses obtained in 3 to 5 different dishes with 12 cells measured per dish. Macrophages were used as detector cells in these experiments because they had the highest sensitivity to the messenger, despite the greater complexity of their response. In the experiment shown, the total activity obtained by summing the three fractions from stimulated cells was 99% of the total activity of unstimu-

lated cells. In 4 such experiments, the corresponding ratio was $110 \pm 7\%$ (mean ± s.e.), confirming rough conservation of total activity over 10 min of stimulation. *b*, Activities appearing in the cytosol of nonstimulated (open bar) or BAPTA-loaded and stimulated cells (closed bar) measured on astrocytoma cells. This experiment was similar to *a* except that the lymphocytes to be stimulated had been loaded with BAPTA by pre-incubation with 50 μM BAPTA/AM for 20 min in HBS with 1.3 mM Ca^{2+} before changing them into the Ca^{2+} -free DPBS (0.5 mM EGTA) and adding PHA. Also, the astrocytoma cells were allowed 15 min to reach their maximal $[\text{Ca}^{2+}]_i$ values. *c*, Activities of the supernatants from lymphocytes stimulated at 10% cytotrit in 1.3 mM Ca^{2+} with 20 μg ml⁻¹ PHA or 50 μM thapsigargin, or held in Ca^{2+} -free medium plus 0.5 mM EGTA without or with PHA, all for 10 min. The cells were then centrifuged. The supernatant was treated with 2 units ml⁻¹ hexokinase and added to macrophages as in *a*. Because each condition was tested on a different day, each $[\text{Ca}^{2+}]_i$ increase was normalized to a matching control obtained the same day from unstimulated cells kept for 10 min in 1.3 mM external Ca^{2+} . These control amplitudes were 24.5, 21, 19 and 19 nM for the four conditions, respectively. The sample activities were all significantly different from their controls (Student's *t*-test gave $P < 0.05$, except $P < 0.09$ for thapsigargin), but not different from each other ($P > 0.2$). The thapsigargin experiments required special care. Control experiments showed that 50 μM thapsigargin was necessary to counteract the strong binding to the high concentration of cells and maintain enough free drug to be bioassayable in the supernatant. To measure the messenger activity, the supernatants from both control and thapsigargin-treated cells were titrated to pH 13 with NaOH to hydrolyse thapsigargin, which contains multiple ester groups. After 20 min, the pH was neutralized to 7.3 with HCl. Control experiments verified that this alkaline treatment completely destroyed 50 μM thapsigargin as judged by its ability to elevate $[\text{Ca}^{2+}]_i$.

pass through gap junctions, it could conceivably have paracrine functions in cell clusters. Also, depletion of Ca^{2+} stores is known to trigger transcription of certain genes^{23,24}, so factors for carrying information from the lumen of Ca^{2+} -sequestering organelles to the cytosol or nucleus may have functions beyond control of Ca^{2+} entry. □

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- Berridge, M. J. *Nature* **361**, 315–325 (1993).
- Tsien, R. W. & Tsien, R. Y. *Rev. Cell Biol.* **6**, 715–760 (1990).
- Irvine, R. F. *FEBS Lett.* **263**, 5–9 (1990).
- Putney, J. W. Jr *Cell Calcium* **11**, 611–624 (1990).
- Hoth, M. & Penner, R. *Nature* **355**, 353–356 (1992).
- Alvarez, J., Montero, M. & Garcia-Sancho, J. *Biochem. J.* **274**, 193–197 (1991).
- Bahnson, T. D., Pandol, S. J. & Dionne, V. E. *J. Biol. Chem.* **268**, 10808–10812 (1993).
- Mason, M. J., Garcia-Rodriguez, C. & Grinstein, S. *J. Biol. Chem.* **266**, 20856–20862 (1991).
- Mason, M. J., Mahaut-Smith, M. P. & Grinstein, S. *J. Biol. Chem.* **266**, 10872–10879 (1991).
- Zweifach, A. & Lewis, R. S. *Proc. natn. Acad. Sci. U.S.A.* **90**, 6295–6299 (1993).
- Ospichuk, Y. & Cahalan, M. *Nature* **359**, 241–244 (1992).
- Chow, S. C. & Jondal, M. *J. Biol. Chem.* **265**, 902–907 (1990).
- Merritt, J. E., Jacob, R. & Hallam, T. J. *J. Biol. Chem.* **264**, 1522–1527 (1989).
- Mason, M. J., Mayer, B. & Hymel, L. J. *Am. J. Physiol.* **264**, c654–c662 (1993).
- Greenberg, S., DiVirgilio, F., Steinberg, T. H. & Silverstein, S. C. *J. Biol. Chem.* **263**, 10337–10343 (1988).

- Alvarez, J., Montero, M. & Garcia-Sancho, J. *FASEB J.* **6**, 786–792 (1992).
- Fulceri, R., Bellomo, G., Gamberucci, A., Romani, A. & Benedetti, A. *Biochem. J.* **289**, 299–306 (1993).
- Kuno, M. & Gardner, P. *Nature* **236**, 301–304 (1987).
- Khan, A. A., Steiner, J. P., Klein, M. G., Schneider, M. F. & Snyder, S. H. *Science* **257**, 815–818 (1992).
- von Tscharnner, V., Prod'homme, B., Baggiolini, M. & Reuter, H. *Nature* **324**, 369–372 (1986).
- Lückhoff, A. & Clapham, D. E. *Nature* **355**, 356–358 (1992).
- Pandol, S. J. & Schoeffield-Payne, M. S. *J. Biol. Chem.* **265**, 12846–12853 (1990).
- Drummond, I. A. S., Lee, A. S., Resendez, E. Jr & Steinhart, R. A. *J. Biol. Chem.* **262**, 12801–12805 (1987).
- Li, W. W., Alexandre, S., Cao, X. & Lee, A. S. *J. Biol. Chem.* **268**, 12003–12009 (1993).
- Lee, H. C., Waiseth, T. F., Bratt, G. T., Hayes, R. N. & Clapper, D. L. *J. Biol. Chem.* **264**, 1608–1615 (1989).
- Jalink, K., van Corven, E. J. & Moolenaar, W. H. *J. Biol. Chem.* **265**, 12232–12239 (1990).
- Peppelenbosch, M. P., Tertoolen, L. G. J., den Hertog, J. & de Laat, S. W. *Cell* **69**, 295–303 (1992).
- Ghosh, T. K., Bian, J. & Gill, D. L. *Science* **248**, 1653–1656 (1990).
- Zhang, H. et al. *J. Cell. Biol.* **114**, 155–167 (1991).
- Tsien, R. Y. & Harootunian, A. T. *Cell Calcium* **11**, 93–109 (1990).
- Alonso-Torre, S. R., Alvarez, J., Montero, M., Sanchez, A. & Garcia-Sancho, J. *Biochem. J.* **289**, 761–766 (1993).

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Depletion of InsP_3 stores activates a Ca^{2+} and K^+ current by means of a phosphatase and a diffusible messenger

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IN non-excitable cells, release of Ca^{2+} from the inositol 1,4,5-trisphosphate (InsP_3)-sensitive store can activate Ca^{2+} entry^{1–3}. Very little is known about the signal mechanism relating store emptying to plasma membrane Ca^{2+} influx. It has been suggested that the signal may be either a diffusible messenger like an inositol phosphate⁴, or the InsP_3 receptor itself, which, by physically coupling to some component of Ca^{2+} entry in the plasma membrane, may link store release to Ca^{2+} entry⁵. The nature of the Ca^{2+} entry pathway is also unclear. Only in mast cells has a very selective Ca^{2+} current been observed after store emptying⁶. Activation of exogenous 5-hydroxytryptamine (5-HT) receptors expressed in *Xenopus* oocytes or direct injection of InsP_3 evokes Ca^{2+} entry activated by InsP_3 pool depletion⁷. Here we investigate the nature of this influx pathway and find a current activated by pool depletion. This has an unusual selectivity in that it is more permeable to Ca^{2+} ions than to other divalent cations (Ba^{2+} , Sr^{2+} or Mn^{2+}). Moreover, a K^+ permeability is also stimulated after pool depletion. The activation of this store depletion current involves both a phosphatase and an unidentified diffusible messenger. Both the Ca^{2+} entry pathway and the activating factors found here may be relevant to pool-depleted Ca^{2+} entry in a variety of non-excitable cells.

Xenopus oocytes are widely used as a model system for Ca^{2+} signalling^{8,9}. Activation of expressed 5-HT receptors that are coupled to InsP_3 , or injection of non-metabolizable analogues of InsP_3 , both activate a similar Ca^{2+} entry pathway through pool depletion⁷. Thapsigargin, a Ca^{2+} -ATPase inhibitor of the InsP_3 store, greatly increases this Ca^{2+} influx¹⁰. Hence emptying

of the InsP_3 -store activates Ca^{2+} entry in oocytes, as in other non-excitable cells⁵. The mechanism of activation of this Ca^{2+} entry pathway is not known. To investigate this, we applied various agents that mimic or interfere with second messenger pathways. The results are summarized in Table 1. None of these agents triggered Ca^{2+} entry alone nor clearly affected either the amplitude or the time course of Ca^{2+} entry in response to 5-HT.

Okadaic acid (a selective inhibitor of phosphatases 1 and 2A¹¹) had a marked effect on Ca^{2+} entry (13 of 14 cells). When applied 10–25 min after 5-HT wash during the decaying phase of Ca^{2+} entry, okadaic acid produced an inward current (8 out of the 14 cells; Fig. 1). This current is similar to the Ca^{2+} influx activated by 5-HT⁷ in that (1), it is sensitive to changes in external Ca^{2+} ; (2), it has a pharmacology similar to the 5-HT current (divalent block $\text{Cd}^{2+} > \text{Co}^{2+}$, for example); and (3), like the 5-HT current, it is blocked by the Ca^{2+} -activated Cl^- channel blocker niflumic acid (data not shown). In five cells in which okadaic acid did not clearly increase the current, the rate of recovery of the current was dramatically altered in that it did not recover as long as okadaic acid was present (10–15 min). On washing, the current recovered fully within 10–15 min. These results demonstrate that a phosphatase is critically involved in Ca^{2+} entry following pool depletion.

If phosphatase activity is the direct link between store emptying and Ca^{2+} entry, then application of okadaic acid without 5-HT pretreatment should activate Ca^{2+} entry. But okadaic acid failed to activate the current at all (5/5 cells), yet the same cells subsequently produced typical Ca^{2+} entry to 5-HT. Moreover, if okadaic acid was applied once the 5-HT current had fully recovered, it evoked no current (5/5 cells), showing that the effect is time-dependent (Fig. 1). This demonstrates that phosphatase block alone is not sufficient to activate Ca^{2+} entry. Phosphatase therefore seems to be modulating the ability of another signal (coming from emptied stores) to evoke Ca^{2+} entry, such that block of the phosphatase makes this signal more pronounced. The time course of this signal may define the time dependence of okadaic acid.

This phosphatase-sensitive signal could be either a kinase that phosphorylates some key component of the Ca^{2+} entry pathway (albeit unusual in that it is not an obvious kinase; Table 1) or a messenger molecule (perhaps broken down by phosphatase) released from store that diffuses to the membrane. Although it is difficult to distinguish between such possibilities, one approach would be to investigate the rate of rundown of the pool-depletion-activated current in excised patches. Run-down would be extremely fast if the signal was a diffusible messenger (within

TABLE 1 Effects of agents that interfere with second messenger pathways on pool-depleted Ca^{2+} entry

Substance	cAMP	cGMP	Ca^{2+}	InsP_4	Phorbol ester	H-7	NDGA	Indomethacin	Genistein
Activate current	no	no	no	no	no	no	no	no	no
Inhibit current	no	no	/	/	yes	no	no	no	weak
Number of cells	6	6	8	5	4	4	3	3	2

Agents that interfere with known second messenger pathways do not affect pool-depleted Ca^{2+} entry. Agents were applied once the Ca^{2+} influx had been triggered by application of 5-HT (to see whether they affected Ca^{2+} entry once it had been evoked) and once again when the current had fully recovered (to see whether the agent alone was sufficient to evoke Ca^{2+} entry). None of the agents tested was able to activate pool-depleted Ca^{2+} entry. Concentrations used for testing: 8-bromo-cAMP, 220 μM ; 8-bromo-cGMP, 78 μM ; Ca^{2+} , 50 nM bolus of 1 μM –2 mM, and InsP_4 , 10 μM ; phorbol ester, 500 nM–1 μM ; H-7, 10–120 μM ; NDGA (nordihydroguaiaretic acid, a lipoxygenase inhibitor), 5 μM ; indomethacin (cyclooxygenase inhibitor), 5 μM ; genistein (tyrosine kinase inhibitor), 30 μM . Tyrosine dephosphorylation has been implicated in Ca^{2+} entry in platelets²¹. *Xenopus* oocytes were isolated and injected with 20 ng cRNA coding for the 5-HT receptor cloned from rat stomach smooth muscle²². Two-electrode voltage-clamp experiments were done as previously described^{22,23}. Cytosolic Ca^{2+} was measured by recording the activity of the Ca^{2+} -activated Cl^- channel^{7,13}. Cells were voltage-clamped at -80 mV; 200 nM 5-HT was bath-applied for 1 min. At least 30 min recovery time was allowed between 5-HT exposures (depending on the recovery of the holding current).

a few seconds), whereas phosphorylation would normally be expected to last longer.

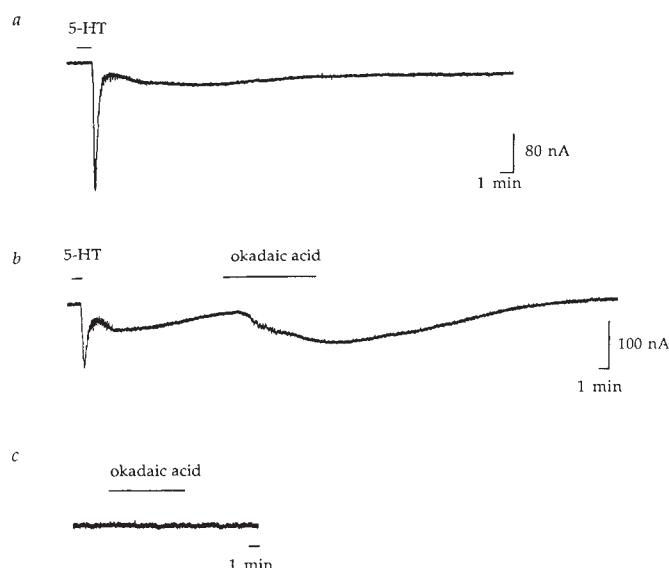
We therefore made cell-attached patch recordings and applied 5-HT to the bath. Exposure to 5-HT increased the current at all potentials (ramps from -120 to 80 mV in 1 s), demonstrating the existence of a diffusible messenger (32/34 patches; Fig. 2). Uninjected cells, injected cells that failed to respond to 5-HT, or perfusion of the 5-HT-sensitive cells with saline (instead of 5-HT), all consistently failed to evoke a current. Heparin, an inhibitor of the InsP_3 receptor, abolishes the whole-cell response to 5-HT⁷, and was found to abolish the increase in both inward and outward patch current evoked by 5-HT (data not shown).

Excision of the patch (now in inside-out configuration, with K^+ -EGTA in the bath) resulted in rapid run-down of the current at all potentials (5/7 cells, reduced by more than 50% in 10 s; Fig. 2). This fast run-down suggests that a cytosolic factor is essential for maintaining pool-depleted current. If true, re-exposing the excised patch to the cytoplasm should enable the current rapidly to recover. Forcing the patch back into the oocyte (patch-cramming¹²) did indeed result in rapid recovery of the current (within 10 s) (4/4 cells; Fig. 2). Because patch-cramming does not return the patch to its original position in the membrane, the increased activity on cramming means that the activating factor is not located at a specific point but is more spread out instead; this is characteristic of a diffusible messenger.

As our currents ran down so quickly on excision and whole-cell dialysis measurements are not feasible on oocytes, it was not

possible to obtain defined bi-ionic conditions, so precluding a systematic study of the selectivity of the pool-depleted current. When Ca^{2+} in the pipette was decreased to 100 μM (from 20 mM), 5-HT did not evoke a clear increase in inward current (in three patches there was no increase, and in two others, only a small increase), showing that the inward current is largely due to Ca^{2+} entry. The outward current was still prominent, indicating a dissociation between Ca^{2+} entry and this outward current. Because oocytes have Ca^{2+} -dependent Cl^- channels¹³, the total patch current reflects Ca^{2+} entry followed by Cl^- channel activation. To isolate the pure Ca^{2+} current, we injected the fast Ca^{2+} -chelator BAPTA. Under these conditions and with Ca^{2+} in the pipette, 5-HT evoked a small but clear increase in inward and outward patch current as well as a positive shift in the reversal potential (Fig. 3). In BAPTA-injected cells, we also tested the selectivity of current activated by store depletion for various divalent cations in the cell-attached mode. Replacing Ca^{2+} with Ba^{2+} or Sr^{2+} in the pipette did not produce a detectable increase in inward current (Fig. 3). Although these results do not rule out some permeability to these divalents, they demonstrate that the influx pathway is more selective for Ca^{2+} . This selectivity profile is unusual for Ca^{2+} channels, but is strikingly similar to a highly selective Ca^{2+} current found in mast cells following pool depletion⁶. A further similarity between these Ca^{2+} entry pathways is their comparable pharmacology^{7,14}. Mn^{2+} , which seems to permeate the same Ca^{2+} influx pathway as Ca^{2+} itself in some cell types^{15–17}, did not produce an inward

FIG. 1 Okadaic acid increases Ca^{2+} entry once the store has been emptied. *a*, Control current to 200 nM 5-HT. Bar indicates 1 min application. *b*, In the same cell, bath application of 30 nM okadaic acid evokes a further increase in current during the declining phase. As 5-HT had been washed out for more than 10 min, okadaic acid is not increasing receptor– InsP_3 coupling. Furthermore, because InsP_3 returns rapidly to basal values (less than 5 min) in oocytes following receptor stimulation²⁴, okadaic acid is not increasing the sensitivity of the InsP_3 receptor to InsP_3 . *c*, Once the current in *b* has fully recovered, application of okadaic acid has no effect (okadaic acid was applied 40 min after the first wash). This shows that okadaic acid works only when Ca^{2+} entry is still activated. The lack of effect of okadaic acid alone argues against a direct action on intracellular Ca^{2+} release or by inhibition of a Ca^{2+} -ATPase. As the okadaic acid current was blocked by Cd^{2+} , it is not acting on the Cl^- channel, which is Cd^{2+} -insensitive⁷. The identical divalent cation block of the okadaic acid current and the store-depletion current (see text) strongly suggests that they are acting on the same Ca^{2+} influx pathway. In *b* and *c*, current scale is the same. METHODS. Oocytes were treated as described for Table 1. Okadaic acid was dissolved in dimethyl sulfoxide (DMSO) (final 0.01%). Although a 10-fold higher volume has been reported to increase InsP_3 -induced Ca^{2+} entry through an unknown mechanism¹⁰, control experiments showed that DMSO had only a weak effect on Ca^{2+} entry. The weak effects may be attributable to a phosphatase inhibition²⁵.



current after 5-HT treatment, suggesting that the pool-depletion current in our patches is not very permeable to Mn^{2+} (data not shown).

Even in the presence of BAPTA, current activated by pool depletion had a reversal potential too negative for a pure Ca^{2+} current. As the only ion with a negative reversal potential and able to carry the outward current under these conditions is K^+ , it seems likely that pool depletion activates an outward K^+ current. Consistent with this are the findings that (1) increasing K^+ in the pipette (in the presence of the non-permeating Sr^{2+}) both increases the inward current and shifts the reversal potential

to the right (Fig. 3); and (2) although excision of the patch into Tris-EGTA or Cs^+ -EGTA solution results in rapid run-down, readdition of K^+ produces a small recovery in the outward current (data not shown). Hence pool-depletion current is carried by both an inward Ca^{2+} current and an outward K^+ current. Although we cannot distinguish whether Ca^{2+} and K^+ permeate the same pathway (as for the ryanodine receptor¹⁸) or different pathways, the fact that we never saw either an inward current or an outward current alone, but always together in our patches, suggests that these pathways may be associated. It is likely that this K^+ flux is important physiologically because it will maintain

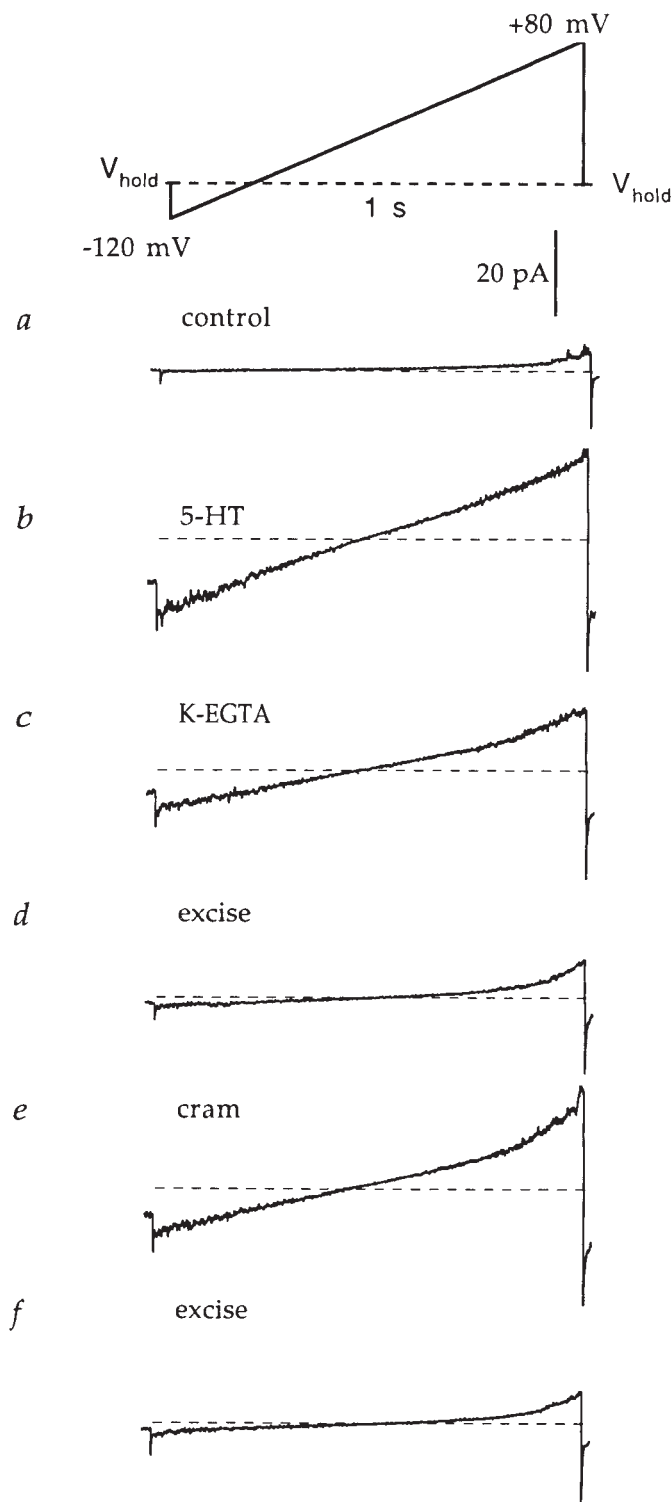


FIG. 2 5-HT increases the patch current through a diffusible messenger. *a*, Control ramp current recorded in cell-attached patches, while recording the cell membrane potential with a microelectrode. The ramp protocol is shown at the top and went from -120 to $+80$ mV in 1 second. The holding potential (V_{hold}) between ramps was -80 mV. In this patch the control ramp reversed at -104 mV. *b*, Bath application of $1 \mu M$ 5-HT increases the current at all potentials. Note the increased noise at low and high potentials. The increase in patch current correlated well with the depolarization of the whole cell. *c*, Bath-applied K^+ -EGTA Ringer's solution reduces the current. This effect is due to Ca^{2+} chelation because reductions were comparable when Na^+ - or Tris-EGTA replaced K^+ -EGTA. These solutions did not contain 5-HT. This increased activity is now independent of receptor stimulation⁷. *d*, The patch was then excised (inside-out). Note run-down of the current. *e*, On cramming the patch back into the cell, the current increased again within seconds. This means that the activity is maintained by a diffusible messenger which is present long after 5-HT wash. Cramming a patch from an uninjected cell into a stimulated cell also produces an increase in patch current. Cramming patches from uninjected or heparin-treated cells back into the cell from which the patch was obtained did not result in such large currents, although occasionally a small increase was observed for the former. The diffusible messenger is therefore not present in the cytosol to any great extent under resting conditions. Because Ca^{2+} injections do not activate Ca^{2+} entry¹³, the messenger is probably released from the $InsP_3$ stores and not synthesized *de novo* in the cytosol after Ca^{2+} release. *f*, Subsequent excision resulted in rapid run-down of the current. The currents in *b-f* reversed at about -30 ± 2 mV.

METHODS. For these experiments, the 5-HT receptor 1c was expressed in oocytes. This receptor evokes large currents, facilitating patch recordings²⁶. Cell-attached and inside-out patch recordings were made as described²⁷. The holding potential of the patch throughout all cell-attached recordings was adjusted to -80 mV (by correcting for the resting membrane potential measured with an intracellular electrode). The holding of the excised patch was also -80 mV. Ramps *b* to *f* were given chronologically, starting 3 min after 5-HT exposure and at 1-min intervals. The pipette contained 90 mM Tris-Cl, 10 mM HEPES, 20 mM $CaCl_2$ (to pH 7.2 with Tris base) and 500 μM niflumic acid to block the Ca^{2+} -activated Cl^- channel²⁸. For *a* and *b*, bathing medium was NFR (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, pH 7.2 with NaOH). In *c-f*, the bath contained K^+ -EGTA Ringer (115 mM KCl, 10 mM HEPES, 1.8 mM EGTA). Patch pipettes had resistances between 0.8 and 1.3 M Ω . Current records were sampled at 4 kHz and low-pass filtered at 300 Hz. They are not corrected for leak and capacitive transients. Dashed lines represent zero current.

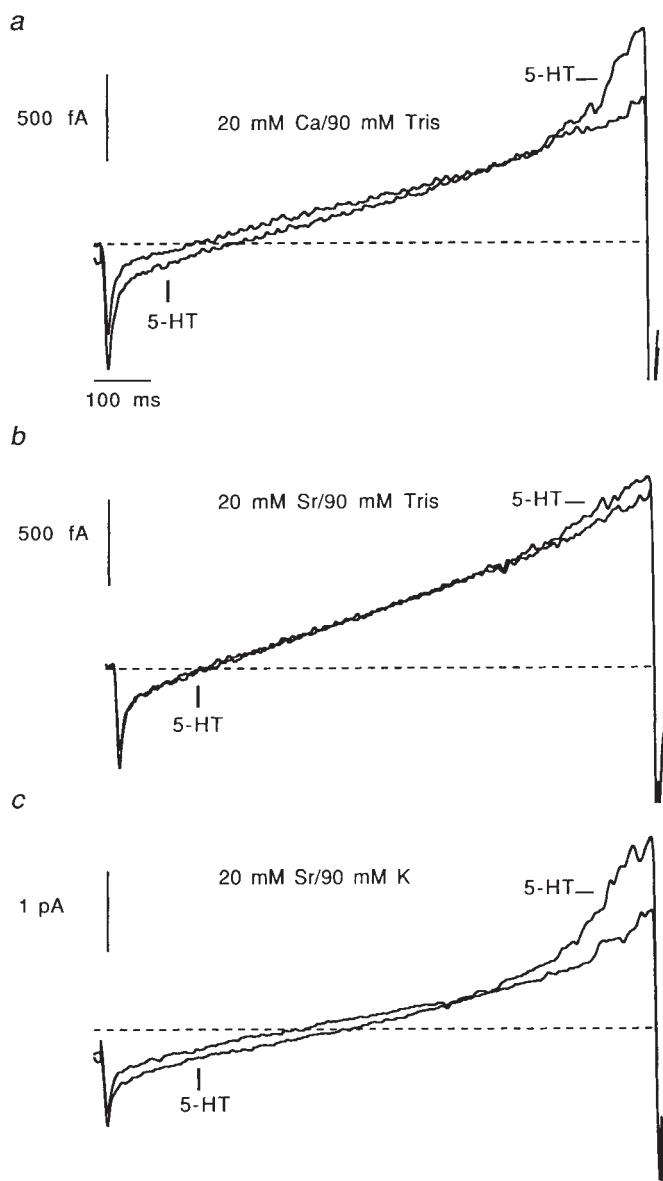


FIG. 3 Current activated by pool depletion is permeable to Ca^{2+} and K^{+} . The selectivity of pool-depleted current was studied with cell-attached patches containing various divalent cations on oocytes injected with BAPTA. **a**, With 20 mM Ca^{2+} in the pipette, there was a small increase in inward current and a shift in the reversal potential to the right. The control ramp reversed at -86 mV; after 5-HT, it reversed at -70 mV. There was no whole-cell depolarization to 5-HT, showing that the Ca^{2+} released from internal stores and Ca^{2+} entry following pool depletion across the whole cell were rapidly buffered by the BAPTA before it could gate the Cl^{-} channel. **b**, Replacing Ca^{2+} in the pipette with Sr^{2+} has no effect on either inward current or reversal potential (-88 mV before 5-HT, and -86 mV after 5-HT). Note the smaller increase in outward current. This may reflect reduction in store-depleted current by certain divalents (including Sr^{2+}), as observed in mast cells¹⁴. Consistent with this was the finding that Ba^{2+} also reduced the outward current (data not shown). The increase in outward current with K^{+} (a permeating ion, see below) may indicate some competition between permeating and poorly permeating cations at an external site of the influx pathway. Recordings in **a** and **b** are from the same cell. **c**, The effect of increased K^{+} in the pipette (90 mM, replacing Tris; the divalent ion was Sr^{2+}) was to increase inward current and shift the reversal potential to the right (-48 mV before 5-HT; -27 mV after 5-HT). Because the cell contained BAPTA, the outward K^{+} flux is not activated by Ca^{2+} . This means that a K^{+} current is activated by pool depletion. Although these patch currents are rather small, this is to be expected as current activated by store depletion is very small in the whole cell⁷. **METHODS.** Cells were first tested for expression of the 5-HT_{1c} receptor by measuring the depolarization with an intracellular microelectrode after addition of $1 \mu\text{M}$ 5-HT. Then they were perfused with an NFR solution in which Ca^{2+} had been reduced to 500 nM or to $1 \mu\text{M}$ free Ca^{2+} (buffered with EGTA) and kept in this medium. Cells were then injected with 100–200 nM 30 mM BAPTA (pH adjusted to 7.0 with KOH), resulting in a final concentration throughout the cell of 5 mM. After 10 min, cells were washed with NFR (1.8 mM Ca^{2+}). Patch recordings were made as for Fig. 2 and were started 1–2 h later to allow for adequate distribution of BAPTA throughout the cell. The pipette solution contained 90 mM Tris-Cl, 20 mM CaCl_2 (or other divalent), 10 mM HEPES and 500 μM niflumic acid (to pH 7.2 with Tris base). Bath solution was NFR. Ramps were given as for Fig. 2, 3–5 min after 5-HT exposure. Current records were sampled at 4 kHz and low-pass filtered at 40 Hz (-3 dB).

(through the hyperpolarization) the large electrical driving force for passive Ca^{2+} entry, thereby enabling more Ca^{2+} to enter the cell.

Our results show that InsP_3 -store emptying activates a Ca^{2+} - and K^{+} -permeable current(s) which occurs through a mechanism involving both a phosphatase and an as-yet-to-be-determined internal diffusible messenger. Because phosphatase block does not activate Ca^{2+} entry in the absence of store release, our results suggest that store emptying releases a molecule which then activates Ca^{2+} entry. This messenger is phosphatase sensitive in that phosphatase block greatly prolongs the duration of Ca^{2+} entry. The molecule may therefore be phosphorylated itself (but is not an inositol phosphate⁷) and inactivated by an okadaic acid-sensitive phosphatase, or it may be a novel kinase and gate Ca^{2+} influx through a phosphorylation/dephosphorylation cycle. Whatever the mechanism, our patch-clamping results indicate that this messenger must be freely diffusible in the cytoplasm. The phosphatase activity may also be controlled by the Ca^{2+} content of the store, having high activity when stores are full and low activity when stores are empty. It is of interest that an okadaic acid-sensitive phosphatase is tightly associated with the plasma membrane¹⁹. As InsP_3 stores are close to the membrane²⁰, the phosphatase may link the store and the membrane, perhaps in conjunction with the mechanism involving the

InsP_3 receptor⁵. This would allow very fine control of the refilling process because both the activator (diffusible messenger) and the inhibitor (phosphatase) would be regulated by the same mechanism (Ca^{2+} store content). Stores more distal to, and therefore not directly coupled with, the membrane may have to regulate the refilling of their Ca^{2+} stores differently. Perhaps InsP_3 stores, depending on their location, use slightly different mechanisms for refilling. □

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- Putney, J. W. Jr *Cell Calcium* **7**, 1–12 (1986).
- Berridge, M. J. & Irvine, R. F. *Nature* **341**, 197–205 (1989).
- Putney, J. W. Jr et al. *FASEB J.* **3**, 1899–1905 (1989).
- Irvine, R. F. *Bioessays* **13**, 419–427 (1991).
- Berridge, M. J. *Nature* **361**, 315–325 (1993).
- Hoth, M. & Penner, R. *Nature* **355**, 353–355 (1992).
- Parekh, A. B., Foguet, M., Lübbert, H. & Stühmer, W. *J. Physiol.* (in the press).
- Parker, I. & Ivorra, I. *Science* **250**, 977–979 (1990).
- Lechleiter, J. & Clapham, D. *Cell* **69**, 283–294 (1992).
- Lupu-Meiri, M., Beit-Or, A., Christensen, S. B. & Oron, Y. *Cell Calcium* **14**, 101–110 (1993).
- Cohen, P. *Trends biochem. Sci.* **15**, 98–102 (1990).
- Kramer, R. H. *Neuron* **2**, 335–341 (1990).
- Miledi, R. & Parker, I. *J. Physiol.* **357**, 173–183 (1984).
- Hoth, M. & Penner, R. *J. Physiol.* **465**, 359–386 (1993).
- Sage, S. O., Merritt, J. E., Hallam, T. J. & Rink, T. J. *Biochem. J.* **258**, 923–926 (1989).
- Merritt, J. E., Jacob, R. & Hallam, T. J. *J. Biol. Chem.* **263**, 1522–1527 (1989).
- Mertz, L. M., Baum, B. J. & Ambudkar, I. S. *J. Biol. Chem.* **265**, 15010–15014 (1990).
- Tinker, A. & Williams, A. J. *J. gen. Physiol.* **100**, 479–493 (1992).
- Kume, H., Takai, A., Tokuno, H. & Tomita, T. *Nature* **341**, 152–154 (1989).

20. Yao, Y. & Parker, I. *Proc. R. Soc. Lond. B* **246**, 269–274 (1992).
21. Vostal, J. G., Jackson W. L. & Shulman, N. R. *J. Biol. Chem.* **266**, 16911–16916 (1991).
22. Foguet, M. *et al.* *EMBO J.* **11**, 3481–3487 (1992).
23. Stühmer, W. *Meth. Enzym.* **207**, 319–339 (1992).
24. Nomura, Y. *et al.* *Molec. Brain Res.* **2**, 113–123 (1987).
25. Zdrojew, J. & Rogers, S. *Life Sci.* **34**, 1967–1975 (1984).
26. Lübbert, H. *et al.* *J. Neurosci.* **7**, 1159–1165 (1987).
27. Hamill, O., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. *Pflügers Arch.* **391**, 85–100 (1981).
28. White, M. M. & Aylwin, M. *Molec. Pharmacol.* **37**, 720–724 (1990).

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Receptor and protein kinase C-mediated regulation of ARF binding to the Golgi complex

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THE formation of constitutive transport vesicles involves the association of non-clathrin coat proteins to transport organelles^{1,2}. Here we report that IgE receptors and protein kinase C (PKC) regulate the GTP-dependent binding of the two coat proteins ADP-ribosylation factor (ARF) and β -COP^{3–5} to Golgi membranes in rat basophilic leukaemia cells. Activation of IgE receptors and PKC prevented the ARF and β -COP dissociation from Golgi membranes that occurs in permeabilized cells in the absence of GTP and potentiated the association-promoting effects of GTP and the G protein activator fluoroaluminate. In contrast, PKC downregulation and PKC inhibition abolished the activity of GTP and fluoroaluminate in promoting ARF binding to the Golgi complex. Studies of ARF binding to isolated Golgi membranes gave similar results. These findings suggest that coat assembly on Golgi membranes, and thus possibly constitutive secretory traffic, is modulated by membrane receptors and second messengers.

In intact rat basophilic leukaemia (RBL) cells, ADP-ribosylation factor (ARF) and β -COP immunolabelling gave a paranuclear Golgi-like staining pattern (see Fig. 3), as previously seen in many other cell types^{6–9}. By contrast, RBL cells permeabilized with streptolysin-O (SLO)¹⁰ in the absence of added GTP had a complete redistribution of both ARF and β -COP to a diffuse cytosolic pattern (Fig. 1a, A) and a partial leakage of the two proteins into the extracellular medium (27% and 7% of total cellular ARF and β -COP, respectively, in 5 minutes) (Fig. 1b, A). As ARF and β -COP behaved very similarly under the experimental conditions tested, only ARF immunofluorescence is shown in Fig. 1. Both ARF redistribution and leakage were completely prevented by guanosine 5'-O-(3-thiotriphosphate) (GTP- γ S, an activator of all GTP-binding proteins) and fluoroaluminate (Al/F, a selective activator of trimeric G proteins¹¹) added at the beginning of the permeabilization period (Fig. 1a, B; b, B). GTP- γ S also caused ARF and β -COP to reassociate, when added 5 minutes after permeabilization (not shown). GTP had similar but less complete effects (Fig. 1a, C, D). These results are compatible with current models proposing that a trimeric G protein is involved in the activation and binding of ARF to Golgi membranes^{12–14}. Antigen-induced activation of IgE receptor for 2 minutes before cell permeabilization prevented

ARF and β -COP redistribution to the cytosol in the absence of added GTP, and enhanced their GTP and fluoroaluminate-dependent association to Golgi membranes (Fig. 1a, E–H). Measurements of ARF leakage gave similar results (Fig. 1b, E–H). The rapid onset of this effect (2 minutes or less) suggested that it might be mediated by a fast-acting second messenger system such as the phospholipase C-PKC pathway, which is known to be coupled to the IgE receptor^{15,16}. Direct activation of PKC by short treatments (2 minutes) with the synthetic diacylglycerol analogue phorbol 12-myristate 13-acetate (PMA) indeed mimicked the effect of IgE receptor stimulation (Fig. 1a, I–N; b, I–N); conversely, prolonged PMA treatments resulting in downregulation of the β isoforms of PKC (see below and Fig. 2)¹⁷ completely abolished antigen activity in preventing both ARF redistribution and leakage through the permeabilization pores (Fig. 1a, O; b, O). Moreover, the specific PKC inhibitor calphostin C (ref. 18, 100 nM for 1 h or 5 μ M for 5 min before permeabilization, under white light), and the kinase inhibitor staurosporine¹⁹ also suppressed these antigen effects (not shown). An additional important consequence of PMA treatments causing β PKC depletion was the complete loss of the ability of GTP and fluoroaluminate (but not GTP- γ S) to promote ARF and β -COP association to Golgi membranes and to prevent ARF leakage in unstimulated cells (Fig. 1a, P–R; b, P–R). Again, similar effects were induced by calphostin C (not shown).

The modulation of ARF binding by PKC was then further characterized by using isolated Golgi preparations⁸ (Fig. 1c). As in the permeabilized cell system, the association of cytosolic ARF to Golgi membranes was increased by GTP- γ S and fluoroaluminate and, less markedly, by GTP. This effect of fluoroaluminate has not been observed by another group⁸, possibly because of differences in experimental conditions or cell types (see legend to Fig. 1), but is in line with the results of Serafini *et al.*⁴. PMA (for 4 min) further stimulated ARF binding in the presence of GTP and fluoroaluminate, whereas calphostin C inhibited the effects of these two agents (Fig. 1c). A prolonged PMA treatment resulting in β PKC downregulation (see below) before homogenization had the same effect as calphostin C (not shown). Although PKC-downregulation experiments must be interpreted with caution because extended exposures to PMA may produce nonspecific or indirect effects, the above results, taken together, demonstrate that PKC regulates the association of ARF to the Golgi complex. Based on the different sensitivities of the effects of fluoroaluminate and GTP- γ S to calphostin C and to β PKC-depleting PMA treatments, it could be speculated that PKC is required for the coupling between the G protein involved in ARF binding and ARF itself. The ARF protein is unlikely to be the target of PKC because it is not phosphorylated normally (R.A.K., unpublished results). Assuming that the relevant PKC substrate is a component of the coat assembly machinery, one could speculate that such substrate might be a subunit of the involved G protein or a regulator of ARF activity such as the recently described guanine nucleotide exchange factor^{20,21}.

In experiments designed to further test whether the observed PMA effects are PKC-mediated, another PKC activator, phorbol 12–13 β -didecanoate (β -PDD), elicited effects similar to those of PMA, whereas its inactive isomer phorbol 12–13 α -didecanoate (α -PDD), did not (not shown). Six PKC isoforms (α , β I, β II, δ , ϵ , ζ) have been described in RBL cells so far; three of them, the α , β I, β II are rapidly and completely downregulated by PMA with characteristic time courses (see Fig. 2 and ref. 17), whereas the δ , ϵ and ζ kinases are not downregulated by overnight treatments with PMA (our manuscript in preparation and ref. 22). At the time point at which fluoroaluminate and GTP have already completely lost their activity in supporting ARF and β -COP binding to Golgi membranes (1 h PMA treatment), only the β I and β II PKCs are downregulated, consistent with the possibility that they may be the isoforms involved in supporting the binding process.

The role of PKC in coat protein cycling was then studied in intact cells challenged with brefeldin A (BFA), a fungal metabolite that reversibly blocks association of coat proteins with Golgi membranes^{20,21,23,24}. Low concentrations of BFA ($0.5 \mu\text{g ml}^{-1}$, inactive in control RBL cells) acquired full effectiveness in releasing ARF and β -COP from the Golgi after a PMA treatment causing β PKC depletion (Fig. 3). Furthermore, in β PKC-depleted cells first treated with high concentrations of BFA to release ARF and β -COP completely from Golgi struc-

tures and then washed free of the toxin, the rate at which coat proteins reassociated on BFA removal was much lower than that seen in parallel experiments in control cells (not shown). This indicates that PKC may play a role in ARF and β -COP binding to the Golgi under physiological conditions. Although the cellular functions and mechanisms of action of ARF and β -COP are still being elucidated, it is clear that these proteins have a central role in coat assembly and intercompartmental vesicular traffic^{7,25}. Our results, suggesting that PKC can control coat

FIG. 1 Antigen and PMA effects on the GTP-dependent association of ARF with the Golgi apparatus. **a**, ARF distribution in SLO-permeabilized cells (immunofluorescence). Control cells (A–D), IgE-primed cells treated with antigen ($0.1 \mu\text{g ml}^{-1}$ BSA–DNP) (E–H, O) for 2 min, and cells treated with 100 nM PMA for 2 min (I–N) or 1 μM PMA for 1 h (O–R), were permeabilized in Tyrode's solution containing 0.8 U ml^{-1} SLO and 5 mM ATP for 5 min at 37°C (ref. 10) in the absence of added guanine nucleotides (A, E, I, O) or in the presence of 1.8 mM MgCl_2 and 100 μM GTP- γ S (B, F, L, P) or 1 mM GTP (C, G, M, Q) (Boehringer–Mannheim) or 50 μM fluoroaluminate (Al/F) (20 mM NaF and 50 μM AlCl_3) (D, H, N, R). Antigen and PMA were also present in the permeabilization medium. Under these conditions SLO permeabilized about 85% of the cells to trypan blue and ethidium bromide (M_r 960 and 395, respectively) and induced a 20–22% loss of lactate dehydrogenase (LDH, M_r 150K) content. These values were not changed by guanine nucleotides, Al/F, PMA and antigen, with the exception of a slight increase in LDH loss (from 22 to 30%) following the prolonged (1 h) treatment with PMA, in some of the experiments. All experiments were done in duplicate on at least five different occasions, with similar results. Scale bar, 10 μm . **b**, ARF leakage from permeabilized cells in the supernatant (immunoblotting and densitometry with Ultrosan XL, LKB). Representative blots are shown for conditions A–D. The optical density (633 nm) values are expressed as per cent of controls. Experiments of ARF leakage by immunoblotting and of ARF localization by immunofluorescence were done in parallel using identical experimental conditions. Values are means of four different experiments run in duplicate; s.d.s were always <15% of the means. Letters under the bars (b) and in the photographs (a) refer to the same treatments. **c**, ARF binding to Golgi membranes *in vitro*. Golgi membranes from RBL cells were incubated with RBL cytosol and, where indicated, with 50 μM GTP- γ S or 1 mM GTP or 50 μM Al/F, either under control conditions (black) or in the presence of 100 nM PMA (oblique lines) or of 5 μM calphostin C activated under white light¹⁸ (grey). In experiments designed to test its specificity of action, calphostin C selectively inhibited the PMA-induced phosphorylation of two proteins in isolated Golgi membranes (manuscript in preparation). **METHODS**. Immunofluorescence: cells were washed, fixed and permeabilized further with 0.05% saponin. They were then incubated with a monoclonal anti- β -COP antibody (M3A5) or with anti-ARF rabbit serum (R5) and then with specific FITC-conjugated secondary antibodies. ARF leakage: the supernatants from permeabilized cell monolayers were precipitated with 10% TCA in 0.4 mg ml^{-1} deoxycholic acid; proteins were subjected to 8–18% SDS–PAGE, transferred to nitrocellulose, immunostained with anti-ARF monoclonal antibody (1D9) and quantified by densitometry. ARF binding: Golgi membranes were isolated from RBL cells as described³³. Cytosol was prepared according to ref. 34. ARF binding to Golgi membranes was assayed as described⁸ with slight modifications consisting of the presence of 100 μM Ca^{2+} and of a shorter incubation time (4 min). The amount of membrane-bound ARF was assessed by immunoblot and densitometry. Values (OD) are expressed as per cent of control; s.d.s were always <10% of the means.

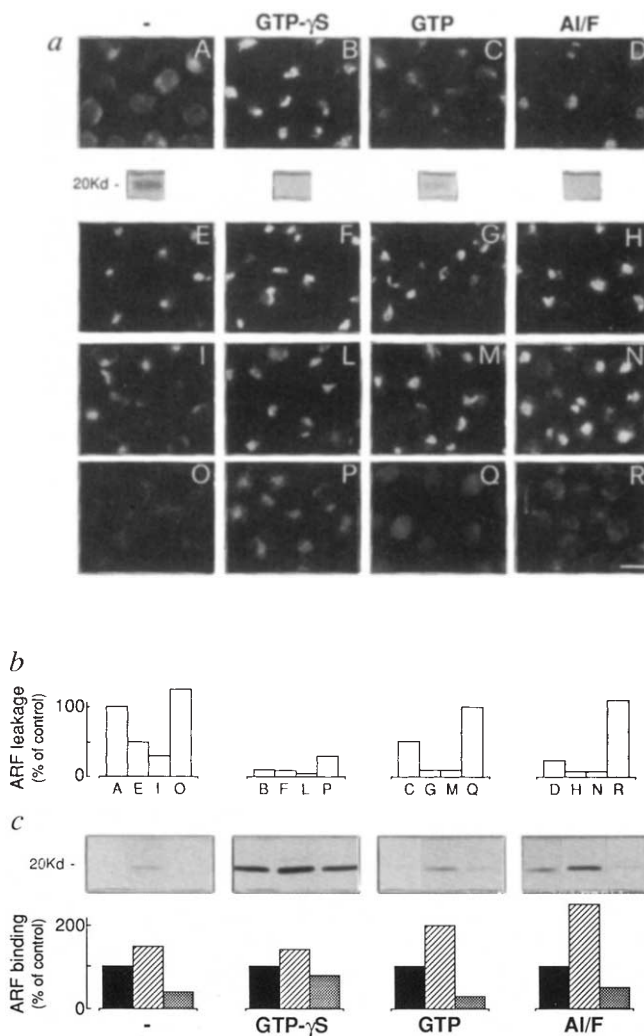
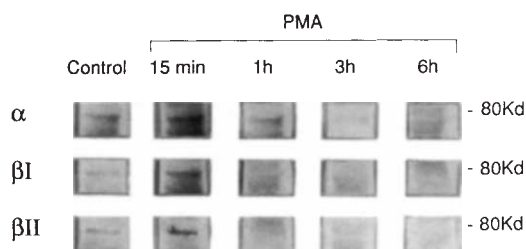


FIG. 2 Differential downregulation of PKC isoforms induced by PMA. Cells were treated for 15 min with 100 nM PMA or for 1, 3 and 6 h with 1 μM PMA. The cellular content of the α , β I and β II PKC isoforms was assayed by lysis in Laemmli buffer (2×10^6 cells per well), resolution by 10% SDS–PAGE and immunostaining with an anti- α PKC monoclonal antibody or with anti- β I and β II rabbit sera. Results shown are representative of four independent experiments in duplicate.



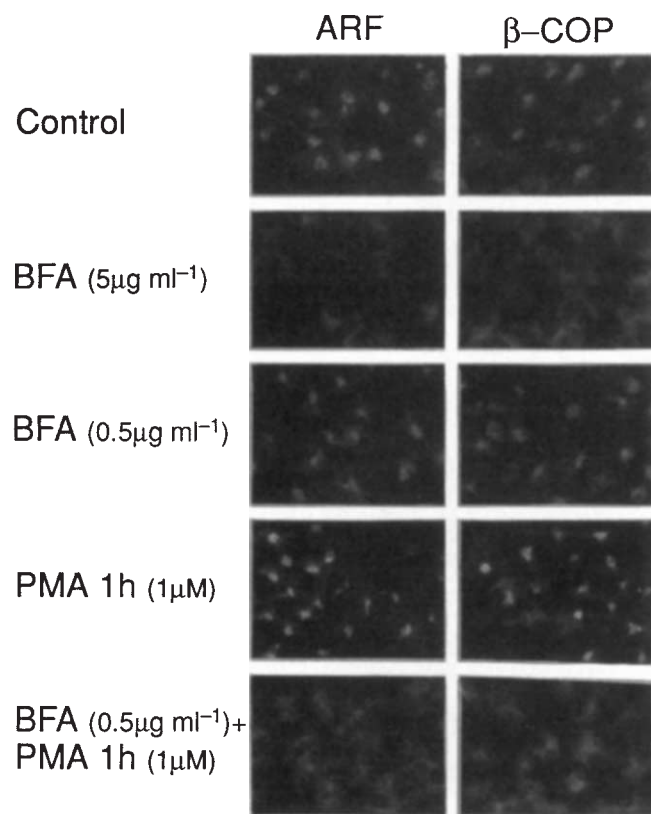


FIG. 3 BFA-induced release of ARF and β -COP from the Golgi complex in intact cells. Effect of prolonged PMA treatments. Control cells or cells treated with $1 \mu\text{M}$ PMA for 1 h were exposed for 5 min to the indicated concentrations of BFA in complete growth medium, then fixed and immunostained with anti ARF and β -COP antibodies as described in Fig. 1a. Scale bar, $10 \mu\text{m}$. Data are representative of four independent experiments done in duplicate.

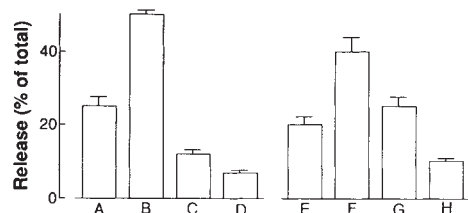


FIG. 4 Effects of PKC stimulation on constitutive release. The release of ^{35}S -GAG chains was measured in RBL (A–D) and in MDCK cells (E–H) under basal conditions (A, E) and in response to 100 nM PMA either alone (B, F), or in combination with $5 \mu\text{g ml}^{-1}$ (C) or $15 \mu\text{g ml}^{-1}$ (G) BFA, or $50 \mu\text{M}$ Al/F (20 mM NaF, $50 \mu\text{M}$ AlCl_3), (D, H). Values are means $\pm$ s.d. of five different experiments run in duplicate. In parallel experiments in RBL cells, the regulated release of serotonin¹⁰ was not affected by PMA (in agreement with published results²⁶) or by BFA and was stimulated by Al/F (not shown). Basal GAG release was inhibited to the same extent (80%) in RBL and MDCK cells by Al/F, whereas BFA was more effective in RBL cells (75% inhibition at $5 \mu\text{g ml}^{-1}$) than in MDCK cells (30% inhibition at $15 \mu\text{g ml}^{-1}$), consistent with the reported low sensitivity of the latter cell line to BFA^{35,36}.

METHODS. Cells were preincubated for 15 min with 1 mM β -D-xyloside, labelled in SO_4 -free medium containing $100 \mu\text{Ci ml}^{-1}$ $^{35}\text{SO}_4$ for 30 min at 37°C , washed rapidly and incubated for 30 min (A–D) or for 15 min (E–H) at 37°C in 4 mM MgSO_4 supplemented Tyrodes' solution in the presence of the above agents. At the end of the release period the supernatants and the cell extracts (0.1 M NaOH for 1 h) were precipitated with cetylpyridinium chloride and $600 \mu\text{g ml}^{-1}$ chondroitin sulphate as a carrier²⁶.

assembly, raise the question as to whether these PKC effects could be correlated with changes in membrane movement through the secretory pathway. The release of glycosaminoglycans (GAGs) can be used as a fluid phase marker of secretion²⁶. Indeed, PMA induced a rapid and marked increase in GAG release (Fig. 4). RBL cells possess both the constitutive and the regulated secretory pathways²⁷, but the latter is not activated by PMA alone²⁸. The PMA-stimulated release was constitutive, as indicated by several lines of evidence: it was inhibited by BFA and fluoroaluminate^{23,29–31}, was accompanied by a rapid change in Golgi size and by a marked increase in the number of non-clathrin-coated buds and transport vesicles and, more importantly, was observed in cells exhibiting only constitutive secretion (MDCK and NIH 3T3); moreover, it differed from regulated secretion based on kinetic and compositional criteria³² (Fig. 4 and manuscript in preparation). These results establish a correlation between the PKC regulation of ARF binding and that of constitutive release; the possible mechanistic link between these two events has now to be elucidated. The possibility that constitutive secretory traffic might be regulated by membrane receptors and second messengers has received little attention so far, in spite of its potential physiological significance. Our results suggest that the association of ARF and β -COP to the Golgi complex might be a key target of cellular signals regulating constitutive vesicle formation and membrane traffic. □

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- Rothman, J. E. & Orci, L. *Nature* **355**, 409–415 (1992).
- Melançon, P., Franzusoff, A. & Howell, K. E. *Trends Cell Biol.* **1**, 165–171 (1991).
- Kahn, R. A. et al. *J. Biol. Chem.* **267**, 13039–13046 (1992).
- Serafini, T. et al. *Cell* **67**, 239–253 (1991).
- Balch, W. E., Kahn, R. A. & Schwaninger, R. J. *Biol. Chem.* **267**, 13053–13061 (1992).
- Stearns, T., Willingham, M. C., Botstein, D. & Kahn, R. A. *Proc. natn. Acad. Sci. U.S.A.* **87**, 1238–1242 (1990).
- Allan, V. & Kreis, T. E. *J. Cell Biol.* **103**, 2229–2239 (1986).
- Donaldson, J. G., Kahn, R. A., Lippincott-Schwartz, J. & Klausner, R. D. *Science* **254**, 1197–1199 (1991).
- Orci, L. et al. *Cell* **64**, 1183–1195 (1991).
- De Matteis, M. A., Di Tullio, G., Buccione, R. & Luini, A. *J. Biol. Chem.* **266**, 10452–10460 (1991).
- Kahn, R. A. *J. Biol. Chem.* **266**, 15595–15597 (1991).
- Donaldson, J. G., Cassel, D., Kahn, R. A. & Klausner, R. D. *Proc. natn. Acad. Sci. U.S.A.* **89**, 6408–6412 (1992).
- Stow, J. L. et al. *J. Cell Biol.* **114**, 1113–1124 (1991).
- Ktistakis, N. T., Linder, M. E. & Roth, M. G. *Nature* **356**, 344–346 (1992).
- Ali, H., Collado-Escobar, D. M. & Beaven, M. A. *J. Immunol.* **143**, 2626–2633 (1989).
- Kawakami, T. et al. *J. Immunol.* **148**, 3513–3519 (1992).
- Huang, F. L., Yoshida, Y., Cunha-Melo, J. R., Beaven, M. A. & Huang, K.-P. *J. Biol. Chem.* **264**, 4238–4243 (1989).
- Bruns, R. F. et al. *Biochem. biophys. Res. Commun.* **176**, 288–293 (1991).
- Azzi, A., Boscoboinik, D. & Hensey, C. *Eur. J. Biochem.* **208**, 547–557 (1992).
- Donaldson, J. G., Finazzi, D. & Klausner, R. D. *Nature* **360**, 350–352 (1992).
- Helms, J. B. & Rothman, J. E. *Nature* **360**, 352–354 (1992).
- Ozawa, K. et al. *J. Biol. Chem.* **268**, 1749–1756 (1993).
- Klausner, R. D., Donaldson, J. G. & Lippincott-Schwartz, J. *J. Cell Biol.* **116**, 1071–1080 (1992).
- Pelham, H. R. B. *Cell* **67**, 449–451 (1991).
- Hosobuchi, M., Kreis, T. & Schekman, R. *Nature* **360**, 603–605 (1992).
- Miller, S. G. & Moore, H.-P. H. *J. Cell Biol.* **112**, 39–54 (1991).
- Burgess, T. L. & Kelly, R. B. A. *Rev. Cell Biol.* **3**, 243–292 (1987).

28. Cunha-Melo, J. R. et al. *J. Immun.* **143**, 2617–2625 (1989).
 29. Strous, G. J., van Kerhof, P., van Meer, G., Rijnboutt, S. & Stoorvogel, W. *J. Biol. Chem.* **268**, 2341–2347 (1993).
 30. Miller, S. G., Carnell, L. & Moore, H. P. *J. Cell Biol.* **118**, 267–283 (1992).
 31. Barr, F. A. et al. *FEBS Lett.* **294**, 239–243 (1991).
 32. Arvan, P. & Castle, D. *Trends Cell Biol.* **2**, 327–331 (1992).
 33. Balch, W. E., Dunphy, W. G., Braell, W. A. & Rothman, J. E. *Cell* **39**, 405–416 (1984).

34. Malhotra, V., Serafini, T., Orci, L., Shepherd, J. C. & Rothman, J. E. *Cell* **58**, 329–336 (1989).
 35. Low, S. H. et al. *J. Biol. Chem.* **266**, 17729–17732 (1991).
 36. Hunziker, W., Whitney, A. & Mellman, I. *Cell* **67**, 617–627 (1991).

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Protein-kinase-A-dependent activator in transcription factor CREB reveals new role for CREM repressors

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HORMONALLY induced increases in cyclic AMP levels induce phosphorylation of the transcription factor CREB at a serine residue at position 133 by protein kinase A (ref. 1), enhancing its ability to activate transcription without affecting its intracellular location or DNA-binding activity. This effect is dependent on a 60-amino-acid region of CREB that contains Ser133 and is termed the kinase-inducible domain (KID)², which also occurs in the CREB-related CREM- α and - β proteins, although these are transcriptional repressors³. Here we show that the KID domain confers a cAMP-inducible increase on the activity of the Q2 activation

domain from CREB and the acidic activation domains from the yeast proteins GAL4 and GCN4. Remarkably, it retains this ability even when attached to a separate polypeptide bound to an adjacent site in the promoter. KID may therefore be the first of a new class of conditional activators that work through other promoter-bound factors to stimulate gene expression in response to hormonal stimuli.

In the process of characterizing CREM-related proteins in the mouse pituitary cell line α T3 (ref. 4), we obtained a novel CREM-like complementary DNA, termed CREM- ϵ , which is homologous to CREB⁵ apart from the absence of a single glutamine-rich domain in CREB, termed Q2 (Fig. 1a). When inserted into a Rous sarcoma virus (RSV) eukaryotic expression vector and tested for transactivation potential in F9 cells, CREM- ϵ was unable to stimulate expression of the cAMP-inducible somatostatin gene, whereas CREB caused a brisk 20-fold activation in response to protein kinase A (Fig. 1b).

The relative inactivity of CREM- ϵ suggested that the glutamine-rich Q2 domain might be critical for CREB activity (Fig. 2a). As predicted, removal of the Q2 region from CREB abrogated both constitutive and protein-kinase-A-inducible activity, indicating that CREM- ϵ inactivity might be ascribed to the absence of the Q2 domain. To determine which portion of the

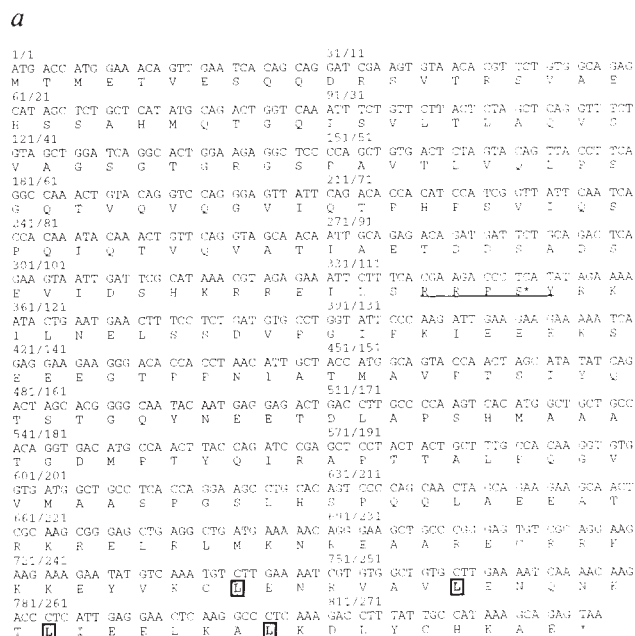
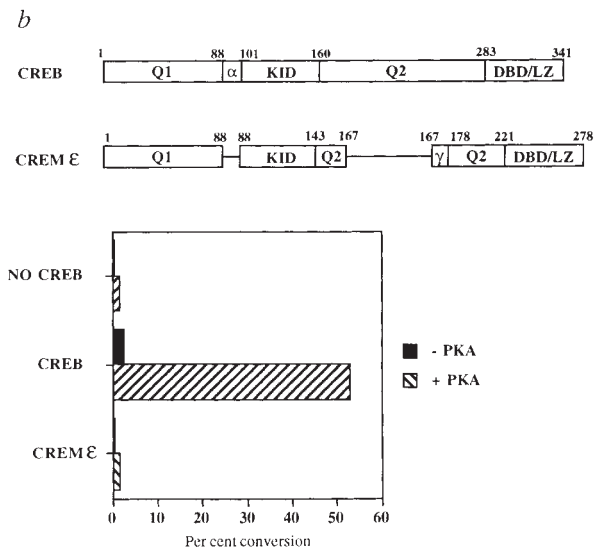


FIG. 1 **a**, Nucleotide and predicted amino-acid sequence (single-letter code) of CREM- ϵ cDNA. The putative protein kinase A (PKA) phosphorylation site is underlined; Ser117 phosphoacceptor is indicated by an asterisk. Leucines in the leucine zipper dimerization domain are boxed. Except for the insertion of the N-terminal glutamine-rich region (found in CREM- τ) and a point substitution at nucleotide 101 (T for C), CREM- ϵ is identical to CREM- α , suggesting that they may be alternative splice products from the same gene. **b**, Comparison of PKA-dependent CREB and CREM- ϵ activities in F9 cells. Top, Schematic comparing homologous regions in CREB and CREM- ϵ . Q1 and Q2, glutamine-rich regions; α , 14-amino-acid α -region; KID, kinase-inducible domain containing the Ser133 phosphoacceptor; DBD/LZ, DNA-binding/leucine zipper domain; γ , unique region in CREM- α , β , ϵ and τ isoforms. Amino-acid



boundaries for each region are indicated. Thin lines show the sequences in CREB that are missing in CREM- ϵ . Graph shows CAT activity (per cent conversion of [¹⁴C]chloramphenicol) derived from a somatostatin CRE-CAT reporter plasmid following transfection into F9 cells with RSV ('NO CREB'), RSV CREB ('CREB'), or RSV CREM- ϵ ('CREM') plasmids plus wild-type ('+ PKA') or mutant PKA ('- PKA') vectors. The mutant PKA contains a Lys → Met substitution at residue 72 in the ATP-binding site, which disrupts catalytic activity¹⁰. CAT assays were normalized to β -galactosidase activity from a cotransfected RSV- β gal plasmid. METHODS. CREM- ϵ cDNA was isolated by polymerase chain reaction (PCR) from an α T3-1 mouse pituitary tumour cell line cDNA plasmid library (gift from M. Perrin) using oligonucleotides corresponding to CREM- α 5' and 3' sequences, 5'-GCATAAGCTTCAATGACCATGGA-AACAGT-3', 5'-GCATAGATCTAATCAACACAGTTACTCTGC-3'. F9 mouse teratocarcinoma cells were maintained and transfected as previously described¹¹. RSV CREM- ϵ was constructed by inserting the subcloned CREM- ϵ PCR fragment into the HindIII/bglII sites of RSV CREB. pGR was used for the RSV expression vector control.

124-amino-acid Q2 region could be critical for activity, we prepared a nested series of Q2-deletion mutants and assayed these in F9 cells. Each CREB mutant was comparably expressed in transfected cells, as determined by western blot analysis (Fig. 2a, bottom). In keeping with the primary structure of Q2, which is uniformly glutamine-rich and hydrophobic, the transactivation potential in Q2 appeared to be evenly distributed over the entire domain; the loss of transcriptional activity in each mutant correlated well with the size of the deletion in Q2.

To test whether Q2 was itself a transactivator, or simply a structural determinant in CREB, we attached the Q2 region (amino acids 160–284) to the yeast GAL4 DNA-binding domain and evaluated the activity of this chimaeric protein (termed GAL-Q2) by transient assay in F9 cells (Fig. 2b). When expressed from an RSV expression plasmid, the GAL-Q2 protein stimulated GAL4 chloramphenicol acetyltransferase (CAT) reporter activity about 15-fold relative to the GAL4 DNA-binding domain alone. Protein kinase A actually decreased GAL-Q2 activity, demonstrating the importance of the Ser 133

protein kinase A site within KID for phosphorylation-dependent induction. By contrast, a GAL-KID expression plasmid, containing the 62-amino-acid KID polypeptide (amino acids 99–161), showed no constitutive activity but induced GAL4-CAT reporter expression 5–6-fold in response to protein kinase A.

The ability of Q2 to function as a constitutive activator prompted us to ask whether other constitutive activators could suppress inactive CREB mutants lacking Q2 but containing the modulatory KID domain (Fig. 3). Remarkably, the acidic GAL4 activator (residues 768–881) restored protein-kinase-A-inducible activity when inserted into the inactive $\Delta 162$ –256 CREB protein. Similar results were obtained when the GAL4 activator was inserted upstream of KID, suggesting that these two domains could indeed function independently. These GAL4-CREB fusion proteins were dependent on the Ser 133 phosphoacceptor site for responsiveness to protein kinase A, however, demonstrating the continued importance of this site for induction (results not shown).

The absence of any spacing constraints between KID and the

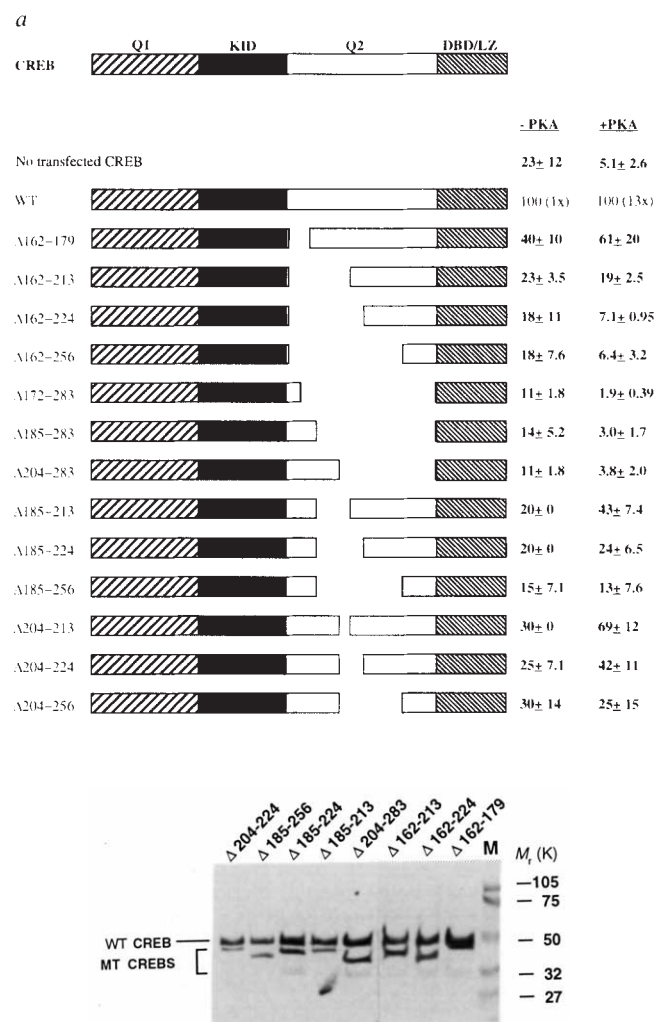
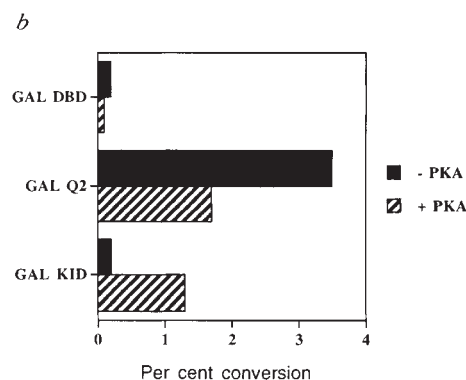


FIG. 2a, Deletional analysis of the CREB Q2 region in F9 cells. Top, block diagram of CREB showing glutamine-rich Q1 and Q2 regions, kinase-inducible domain (KID), and the DNA-binding/leucine zipper dimerization domains (DBD/LZ). CREB sequence deletions are indicated on the left by inclusive endpoints. WT, wild-type CREB control. Results (right) are expressed as percentage of wild-type CREB activity under induced (+PKA) and non-induced (–PKA) conditions as averaged ($\pm$ s.e.) from a minimum of two independent experiments for each con-



struct. Average PKA induction for wild-type CREB was 13-fold ($13 \times$) over all experiments. CAT was assayed in extracts prepared from F9 cells transfected with a somatostatin CRE–CAT reporter, RSV CREB expression plasmids, and RSV- β gal internal control plasmid as described in Fig. 1. Bottom, mutant CREB polypeptides are expressed at comparable levels in transfected cells. Western blot analysis using CREB antiserum 244 on crude nuclear extracts of α T3-1 cells transfected with mutant CREB expression vectors. CREB sequence deletions indicated over each lane by inclusive endpoints. M, molecular size markers, with M_s (in thousands) indicated to the right. WT CREB, endogenous CREB protein recovered from nuclear extracts of transfected cells. MT CREBs, mutant CREB polypeptides expressed from individual effector plasmids. b, CREB Q2 and KID regions are independent activators. Representative CAT assay showing activity derived from a somatostatin reporter plasmid containing five GAL4 binding sites ($5 \times$ GAL-CAT) in place of the CRE. Graph compares activity of SV40 expression vectors containing GAL4 DNA-binding domain alone ('GAL DBD'), fused to the CREB Q2 region (amino acids 160–284; GAL Q2), and to the KID domain (amino acids 99–161; GAL KID). Data are expressed as per cent conversion 14 C-chloramphenicol to acetylated forms.

METHODS. a, α T3-1 pituitary cells were transfected with 20 μ g of each CREB expression plasmid, and nuclear extracts were prepared from these cells 20 h later. CREB antiserum 244, raised against a synthetic CREB peptide (amino acids 128–151) was used to examine mutant CREB expression by western blot analysis. b, CREB Q2 deletion mutants were constructed by *Bal*31 exonuclease digestion followed by blunting with Klenow fragment and ligating with *Xho*I linkers (5'–CCTCGAGG–3'). In-frame Q2 deletion mutants were analysed for activity after cloning into parent RSV CREB expression vector. GAL4 expression vectors were assembled using pSG424 parent vector¹². For transfection, 5 μ g effector and 10 μ g GAL4-CAT reporter plasmid were used. Each assay was repeated at least three times.

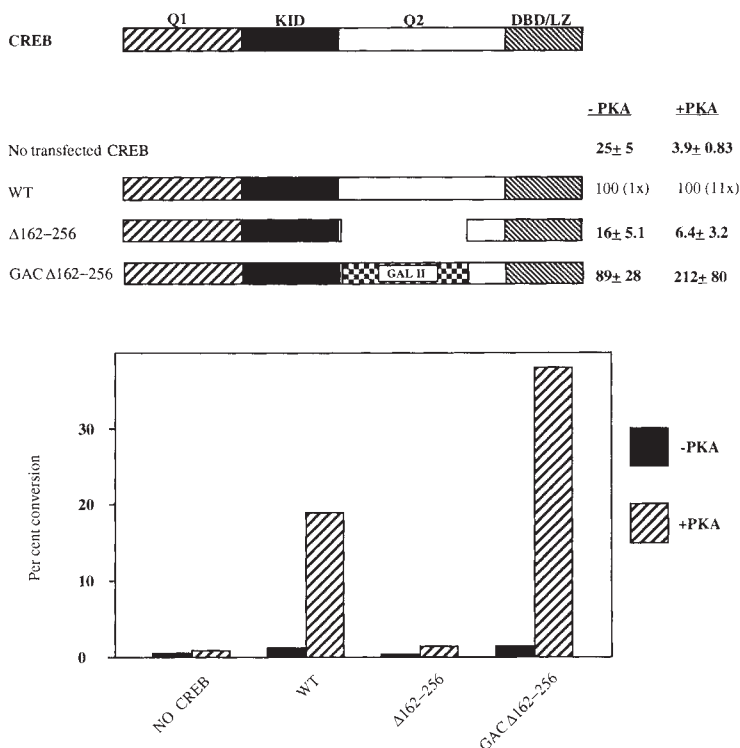


FIG. 3 GAL4 acidic activation domain suppresses a CREB Q2 deletion mutation *in cis*. Top, diagram of CREB showing glutamine-rich Q1 and Q2 regions, the kinase-inducible domain (KID), and the DNA-binding domain/leucine zipper (DBD/LZ). WT, wild-type CREB; Δ162-256, CREB mutant containing Q2 deletion as indicated in diagram; GAC Δ162-256, CREB containing GAL4 acidic activation domain II (amino acids 768-881) inserted into Δ162-256 CREB mutant. Data (averaged for 3 independent experiments ± s.e.) expressed as percentage of wild-type CREB-dependent CAT activity for the induced (+PKA) and non-induced (-PKA) state. 11 ×, average induction of wild-type CREB by PKA. Bottom, graph showing representative CAT assay in F9 cells using constructs described above. Per cent conversion represents acetylation of ^{14}C -chloramphenicol.

METHODS. Transfections of F9 cells and CAT assays were done as described for Fig. 1 after normalizing to the activity of cotransfected RSV-βgal plasmid. GACΔ162-256 was constructed by removing an *Xba*I/*Bam*HI fragment containing the GAL4 acidic activated domain II (amino acids 768-881) from pGAD (ref. 13), blunting the ends with Klenow fragment, attaching on *Sall* linkers (5'-GCTCGACG-3'), and cutting with *Sall*. The resulting fragment was ligated into the *Xho*I site of RSV CREB Δ162-256.

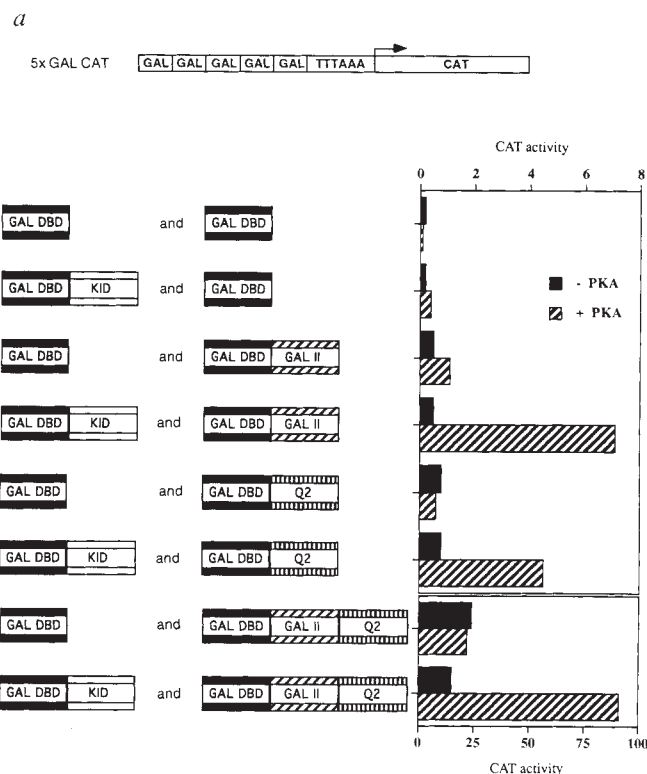
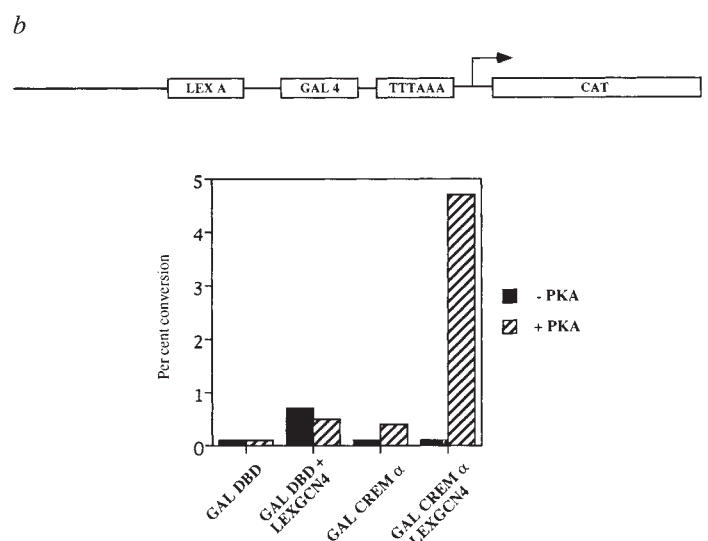


FIG. 4 a, The CREB kinase-inducible domain (KID) can cooperate with constitutive activators *in trans* to support PKA-dependent transcription. Representative CAT assay showing 5 × GAL CAT reporter activity derived from F9 cells following cotransfection with expression vectors containing the N-terminal 147-amino-acid GAL4 DNA-binding domain alone (GAL DBD), fused to CREB Q2 region (GAL Q2), to KID (GAL KID), to the GAL4 acidic activation domain II (GALII), or Q2 + GALII activators together (GALII Q2). A separate graph scale was used for the GALII Q2 assay, which was necessary because of the higher relative activity of this fusion protein. b, CREM-α functions as a PKA-dependent modulator when



juxtaposed to a constitutive activator. Transient assay in F9 cells using LexA-GAL4 CAT reporter, plus SV40 expression vectors containing GAL4 DNA-binding domain alone (Gal DBD), or fused to CREM-α cDNA (GAL CREM-α). Lex GCN4, RSV expression vector containing LexA DNA-binding domain fused to yeast GCN4 acidic activation domain (gift from R. Evans). Graph shows per cent conversion from a representative CAT assay after normalizing to the activity derived from cotransfected RSV-βgal plasmid. pGR was used for the RSV expression vector control. Each assay was repeated at least three times.

METHODS. a, Equal amounts of each expression plasmid (2.5 μg each) were mixed for transfection as indicated in the diagram on the left. GAL DBD control had 5 μg plasmid only. Data shows relative CAT activity after normalizing to activity from cotransfected RSV-βgal plasmid. GBD GALII is pGXGAL-II (gift from M. Ptashne¹⁴). b, CREM-α cDNA was obtained by ligating appropriate fragments from CREM-ε cDNA and another CREM isoform (P.B., unpublished data) to generate a cDNA with predicted amino-acid sequence identical to CREM-α. GAL CREM-α was constructed by insertion into the GAL4 expression vector pM2 (gift from I. Sadowski¹⁵).

GAL4 activator *in cis* suggested that these domains might also cooperate *in trans* (Fig. 4a). To test this model, we used an expression vector containing the GAL4 activator fused to the GAL4 DNA-binding domain (GAL II). Although both GAL KID and GAL II expression vectors displayed marginal activity when tested separately, they produced a 14-fold response to protein kinase A when transfected together. Similarly, cotransfection of GAL KID plus GAL Q2 expression plasmids elicited an almost 6-fold induction in response to protein kinase A, demonstrating the ability of KID to modulate both acidic and glutamine-rich activators *in trans*. The KID region had comparable effect when assayed with a much stronger constitutive activator (GAL II Q2), further indicating that absolute induction by protein kinase A depended on the type of activator in juxtaposition.

The autonomy of KID *in vivo* prompted us to investigate whether the KID-containing family of CREM repressors might correspondingly stimulate protein-kinase-A-dependent transcription when *in trans* to certain constitutive activators. We used the CREM- α protein, which lacks both glutamine-rich Q1 and Q2 regions⁶, while retaining the KID modulator (Fig. 4b). To eliminate background contributions from endogenous CRE-binding proteins in F9 cells, we attached the GAL4 DNA-binding domain to CREM- α . To provide a constitutive activator, we created a LexA-GCN4 fusion plasmid containing the acidic GCN4 activator domain fused to the bacterial LexA DNA-binding domain. When assayed in F9 cells, the GAL CREM- α effector plasmid showed very low constitutive and protein-kinase-A-inducible activities on a GAL4-LexA CAT reporter plasmid; the LexA-GCN4 fusion protein supported constitutive but not protein-kinase-A-dependent reporter expression. On cotransfection of the GAL CREM- α and LexA-GCN4 effector plasmids, however, CAT reporter activity increased about 40-fold in response to protein kinase A, demonstrating the ability of the previously characterized CREM- α repressor to promote cAMP-dependent transcription. Mutant effector plasmids containing a Ser→Ala substitution at the protein kinase A phosphoacceptor site were unable to synergize with Lex-GCN4, however, demonstrating the importance of this site for protein kinase A induction (result not shown).

From these experiments, we conclude that the CREM/CREB proteins that are responsive to protein kinase A are organized in a modular fashion, with constitutive and modulatory domains working synergistically to stimulate transcription. The CREB-type proteins can stimulate transcription independently by virtue of a glutamine-type activator which synergizes *in cis* with the modulatory KID domain. The CREM proteins, however, may only confer cAMP inducibility on promoters containing sites for other constitutive activators. An AP-2 site seems to be critical for CRE-mediated activity on the human tissue plasminogen activator (tPA) promoter in HeLa cells, for example⁷. Moreover, NF-1 and CRE sites juxtaposed on the rat tPA promoter are essential for full cAMP response in granulosa cells⁸, and NF-1 protein may synergize with CREM to support cAMP-dependent expression of the tPA gene there.

Our results suggest that CREM can stimulate protein-kinase-A-dependent transcription by synergizing *in trans* with constitutive activators. In the absence of such factors, however, the CREM proteins may actually repress protein-kinase-A-induction by competing for promoter occupancy with 'autonomous' activators like CREB. Other repressor-activator cognate pairs such as FosB and Δ FosB (ref. 9) may be similarly distinguished by the absence of an activator that can be provided *in trans* to reconstitute function.

Note added in proof: Following submission of this manuscript a cDNA for CREM- ϵ has been reported independently¹⁶. □

- Windle, J. J., Weiner, R. I. & Mellon, P. M. *Molec. Endocr.* **4**, 597–603 (1990).
- Gonzalez, G. A. et al. *Nature* **337**, 749–752 (1989).
- Foulkes, N. S., Borrelli, E. & Sassone-Corsi, P. *Cell* **64**, 739–749 (1991).
- Medcalf, R. L., Rüegg, M. & Schleuning, W.-D. *J. biol. Chem.* **265**, 14618–14626 (1990).
- Ohlsson, M., Leonardsson, G., Jia, X.-C., Feng, P. & Ny, T. *Molec. cell. Biol.* **13**, 266–274 (1993).
- Nakabeppu, Y. & Nathans, C. *Cell* **64**, 751–759 (1991).
- Huggenvik, J., Collard, M., Stofko, R., Seasholtz, A. & Uhler, M. *Molec. Endocr.* **5**, 921–930 (1991).
- Hagiwara, M. et al. *Cell* **70**, 105–113 (1992).
- Sadowski, I. & Ptashne, M. *Nucleic Acids Res.* **17**, 7539 (1989).
- Chien, C., Bartel, P. L., Sternglanz, R. & Fields, S. *Proc. natn. Acad. Sci. U.S.A.* **88**, 9578–9582 (1991).
- Carey, M., Lin, Y., Green, M. R. & Ptashne, M. *Nature* **345**, 361–364 (1990).
- Sadowski, I., Bell, B., Broad, P. & Hollis, M. *Gene* **118**, 137–141 (1992).
- Laoidé, B., Foulkes, N., Schlotter, F. & Sassone-Corsi, P. *EMBO J.* **12**, 1179–1191 (1993).

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Fission yeast *wee1* protein kinase is not required for DNA damage-dependent mitotic arrest

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CHECKPOINTS maintain the dependency relationships between discrete events in the cell cycle (for example, ensuring mitosis does not occur before DNA replication is complete)^{1,2}. In *Schizosaccharomyces pombe*, mitotic checkpoints monitor DNA synthesis³ and the presence of DNA damage^{4,5}. The replication-dependent mitotic checkpoint prevents mitosis by inactivating p34^{cdc2} kinase³. The mechanism by which the DNA damage checkpoint interacts with the mitotic machinery is distinct from that used by the replication checkpoint^{4,5}. The activity of p34^{cdc2} is controlled, in part, by the *wee1* protein kinase⁶, which inactivates *cdc2* through phosphorylation at tyrosine-15 (ref. 7). Here we report normal mitotic arrest after DNA damage in *S. pombe* cells in which the *wee1* gene is defective or missing. We suggest why these findings contradict a recent report⁸ which suggested that the *wee1* gene product was required for DNA damage-dependent mitotic arrest.

S. pombe cells lacking functional *wee1* gene product (p107^{wee1}) are sensitive to the killing effects of ultraviolet and gamma irradiation^{4,8}. But, *cdc2.1w* cells (an allele of the *cdc2*⁺ gene largely insensitive to *wee1* inhibition⁹) are not radiation sensitive⁴. This suggests that the radiation sensitivity of *wee1* mutant cells is not a consequence of *wee1* kinase interacting with the mitotic apparatus. To establish the role of p107^{wee1} in DNA damage-dependent mitotic arrest, we examined the effects of irradiation on progression into mitosis in synchronous cultures of *wee1.50* cells and *wee1::LEU1* deletion mutants⁹. The *wee1.50* mutant is temperature-sensitive for *wee1* function¹⁰. At 36 °C p107^{wee1} is inactive and cells enter mitosis at a reduced size¹⁰. A synchronous culture¹¹ of *wee1.50* cells showed a peak of mitosis at 45 min at the restrictive temperature. In a sample irradiated with 50 Gy at time 0, mitosis peaked at 90 min. In a sample irradiated at 20 min, mitosis peaked at about 105 min (Fig. 1A). The mitotic delays (comparing irradiated and unirradiated samples) were therefore 45 and 60 min. But the time between irradiation and mitosis remained roughly constant at 90 and

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- Gonzalez, G. A. & Montminy, M. R. *Cell* **59**, 675–680 (1989).
- Gonzalez, G. A., Menzel, P., Leonard, J., Fischer, W. H. & Montminy, M. R. *Molec. cell. Biol.* **11**, 1306–1312 (1991).
- Foulkes, N. S. & Sassone-Corsi, P. *Cell* **68**, 411–414 (1992).

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85 min. The observed delay did not result from partial $p107^{wee1}$ activity immediately after the temperature shift, as the second mitosis of a synchronous culture could be delayed by irradiation at first interphase (Fig. 1B).

Very similar results were obtained when a $wee1^+$ control culture was irradiated immediately preceding mitosis (Fig. 2a). The mitotic delay was very dependent on the time of irradiation, but the time between irradiation and the subsequent mitosis remained constant at about 95 min, irrespective of the time of irradiation. In a synchronous culture of $rad17.d$ (null) cells there was no significant mitotic delay, irrespective of the time of irradiation (Fig. 2b). $rad17$ mutant cells lack radiation-induced mitotic delay^{4,5}. These results were not due to any peculiarities in the experimental procedures since very similar results were obtained

in $wee1$ deletion mutant cells⁹ synchronized in a completely different way (Fig. 3).

In contrast to our findings, Rowley *et al.*⁸ reported that a synchronous culture of $wee1.50$ cells did not appear to manifest a significant mitotic delay in response to 100 Gy ionizing radiation (see Fig. 3a in ref. 8). These authors concluded that $p107^{wee1}$ functions at the radiation checkpoint. Examination of their experimental procedure shows that there are two possible reasons why they were misled. (1) Only one time of irradiation was used. They irradiated synchronized $wee1.50$ cells 75 min before the peak of septation (their marker for mitosis) in the unirradiated sample. When the irradiated sample (peak of septation 105 min) was compared to the unirradiated sample, the apparent mitotic delay was 30 min. We have established that

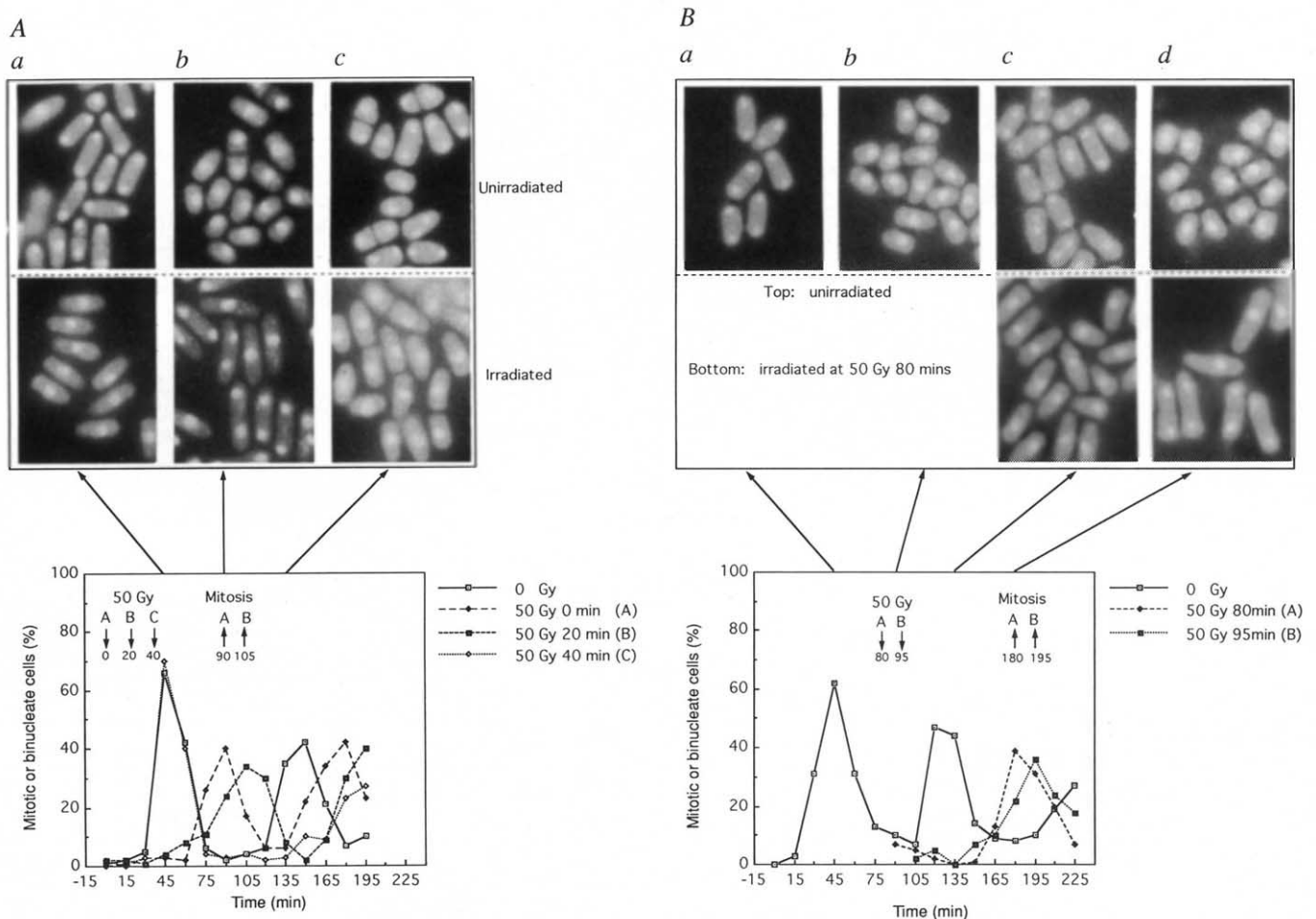


FIG. 1 $wee1.50$ cells delay mitosis by 90 min in response to 50 Gy ionizing radiation. The mitotic profile of synchronous $wee1.50$ cells is shown after irradiation with 50 Gy ionizing radiation. A, Irradiation at 0, 20 and 40 min to examine the delay of the first synchronous mitosis. The period of mitotic delay is dependent on the time (relative to mitosis in the unirradiated cells) at which the dose is delivered. When the dose is delivered during mitosis (dotted line C), the second synchronous mitosis is inhibited. a–c, Photographs of the actual cells scored (top unirradiated, bottom irradiated, 0 min). a, At 45 min, irradiated cells show no evidence of mitosis, unirradiated cells enter mitosis synchronously. b, At 90 min, the irradiated cells show a peak of mitosis, unirradiated cells are in interphase. c, At 135 min, the irradiated cells are in interphase, unirradiated cells enter the second synchronous mitosis. B, Irradiation at 80 and 95 min to examine the delay of the second synchronous mitosis. a–d, Photographs of the cells scored (top unirradiated, bottom irradiated 80 min). a, 45 min, first synchronous mitosis. b, at 90 min, interphase. c, At 135 min irradiated cells show no evidence of mitosis whereas unirradiated cells enter the second synchronous mitosis. d, At 180 min the irradiated cells are dividing, unirradiated cells

are in the second interphase. Taken together, these data demonstrate that the peak of mitosis is inhibited for 90–95 min (90, 85, 100 and 100 min) in $wee1$ deficient cells after 50 Gy ionizing radiation.

METHODS. Synchronous cells prepared as described in ref. 11. A log culture (27 °C, yeast extract) was collected, resuspended in 1 ml yeast extract and layered onto a 7.5%–30% lactose gradient (in yeast extract) and centrifuged for 8 min at 500g. This fractionates the cells by size. Recently-divided cells are small and were extracted from the top of the gradient. These cells were collected and resuspended in yeast extract. A, The sample was divided into four and incubated at 36 °C. One fraction was irradiated at time zero (while being maintained at 36 °C), a second at 20 min and a third at 40 min. A control sample was left unirradiated. B, The entire sample was incubated at 36 °C. At 80 and 90 mins, aliquots were removed and irradiated. In all cases, samples were removed at 15 min intervals, fixed in methanol⁴, examined by microscope after staining with DAPI⁴ and scored for mitotic and binucleate cells. We have not included cells with a clearly visible septum under these staining conditions. Irradiation was in a Gammacell 1000 ¹³⁷Cs source. Dose rate, 12 Gy min⁻¹.

there is a minimum time between irradiation and the onset of mitosis which is similar in *wee1⁻* and *wee1⁺* cells. At 36 °C after 100 Gy ionizing radiation this period is about 105 min (Fig. 4b). This is entirely consistent with the synchronous culture data reported by Rowley *et al.* It is likely that, had they irradiated their cells closer to mitosis, they would have observed a delay similar to that which we have shown in Fig. 1. (2) Rowley *et al.* did not subject a wild-type control to similar treatment. Instead, these authors compared the timing of septation in irradiated *wee1.50* synchronous cells at 36 °C with a control that was irradi-

ated, but maintained at room temperature (conditions at which p107^{wee1} is active). Septation in this '*wee1⁺* control' peaked at about 240 min after irradiation. The authors conclude that this supported their view that p107^{wee1} was required for mitotic delay after irradiation.

We have shown that mitotic delay is increased at lower temperatures in wild-type cells (Fig. 4a). At 22 °C most wild-type cells did not enter mitosis for about 195 min after irradiation. Again, this is consistent with the data presented by Rowley *et al.* who were thus misled by comparing the delay observed in

FIG. 2 Checkpoint proficient and deficient controls. *a*, In a wild-type culture the mitotic delay at 36 °C in response to 50 Gy ionizing radiation is dependent on the time of irradiation. The time between irradiation and the peak of mitosis is, ~95 mins (105, 90 and 90 mins). *b*, In *rad17* deletion mutant cells (radiation checkpoint deficient^{4,5}) there is no mitotic delay in response to 50 Gy ionizing radiation. The method used to prepare cells and score samples was identical to that in Fig. 1. The degree of synchrony in *wee1.50* cells is slightly better as these cells enter mitosis earlier and at a small cell size at 36 °C¹⁰.

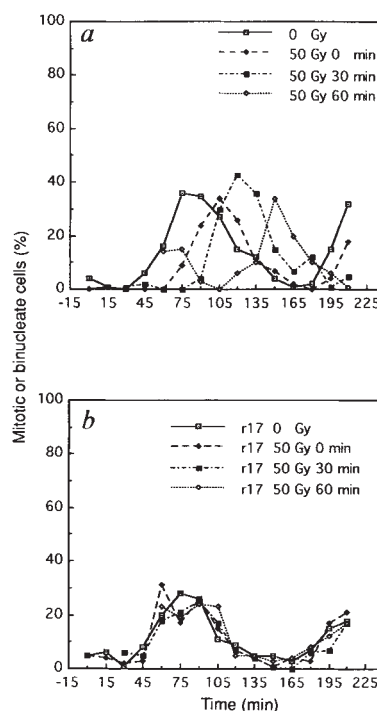
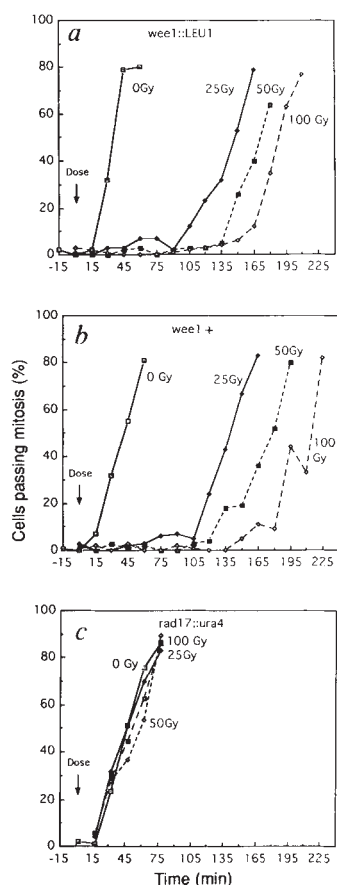


FIG. 3. Radiation induced mitotic delay in *wee1::LEU1* mutant cells. A genetic method of synchronizing cells was used for this experiment. In *a* (*wee1::LEU1* cells) and *b* (*wee1⁺* cells) the *cdc2.33* allele was used to provide a genetically synchronized culture. At doses of 25, 50 and 100 Gy the duration of the mitotic delay was very similar in *wee1*-proficient and *wee1*-deficient cells. *c* (*rad17::ura4* cells), no delay is evident in *rad17* (null) cells which have been genetically synchronized using the *cdc25.22* mutant.

METHODS. A *cdc2.33 wee1::LEU1* double mutant, a *cdc25.22 rad17::ura4* double mutant and a *cdc2.33* control culture were nitrogen starved overnight to synchronize cells before 'start'. Cells were collected and incubated at 35 °C for 4 h in complete media. This procedure ensures that the cells are blocked at the G1/S transition¹². The cultures were shifted to 27 °C and irradiated after 30 min. After irradiation (time zero), samples were taken, fixed in methanol, DAPI stained and scored for cells that had unambiguously passed mitosis (mitotic, binucleate and septated cells). This method of scoring gives the clearest graphical representation of the results. A minimum of 100 cells per time point were scored. The delay can be estimated as the time taken for 50% of cells to pass mitosis. These observations have been repeated on three occasions, and always produced results where the delays observed in *wee1⁻* cells are equivalent to those seen in the *wee1⁺* control. *rad17* null cells have lost, in addition to the DNA damage checkpoint, the dependency relationship between DNA synthesis and mitosis^{4,5} making them unsuitable for G1 block and release experiments (mitosis occurs almost immediately, presumably without prior completion of DNA synthesis). In the *rad17* experiment the block was imposed at G2 by the *cdc25.22* allele and cells were irradiated at time 0.

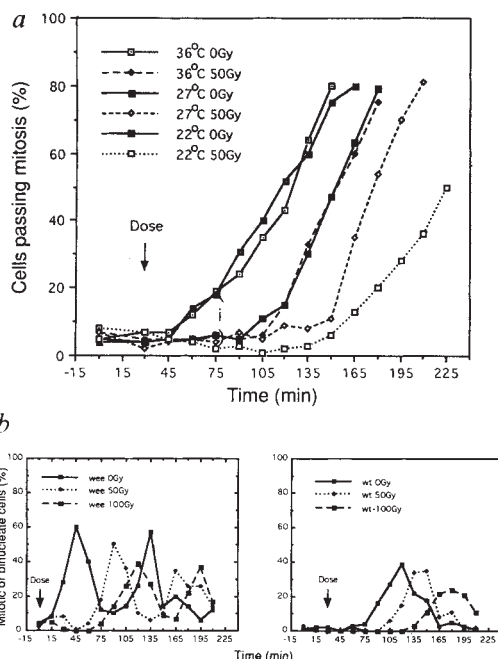


FIG. 4 The duration of mitotic delay after ionizing radiation is dependent on temperature and dose. *a*, Samples from a wild-type synchronous culture (initially grown at 27 °C) were irradiated with 50 Gy at time 30 min. One irradiated and one unirradiated sample were incubated at either 36 °C, 27 °C or at room temperature (22 °C). Samples were taken at 15-min intervals, fixed in methanol and stained as described in Fig. 1. The scoring procedure in this experiment is equivalent to that described for Fig. 3, as this gives the clearest graphical representation of the data on a single graph. At decreasing temperature, the mitotic delay increases dramatically. *b*, Response of *wee1.50* (left) and wild-type (right) cells to 50 and 100 Gy radiation. When the dose was doubled (from 50 to 100 Gy) the duration of the mitotic delay was increased to similar extents in both *wee1*⁻ and wild-type cells. The method used is equivalent to that in Fig. 1.

their '*wee1*⁺ control' (room temperature) with that observed at the restrictive temperature for *wee1* function (36 °C).

If p107^{*wee1*} was responsible for cell-cycle arrest after irradiation, *cdc2.1w* cells (p34^{*cdc2*} insensitive to the *wee1* kinase⁹) should, like *wee1*⁻ cells, be radiation sensitive. But *cdc2.1w* cells are not sensitive to either gamma⁸ or ultraviolet⁴ irradiation. In all other respects (cell size at division¹³, increased mitotic catastrophe during logarithmic growth^{13,14} and virtual inviability in the presence of the checkpoint *rad* mutants⁴ (*rad1*, *rad3*, *rad9* and *rad17*)) the *cdc2.1w* mutant appears to mimic the *wee1*⁻ phenotype. The demonstration that *wee1* mutants are radiation sensitive^{4,8}, and that this is not a consequence of the interaction with the mitotic apparatus, provides clear evidence of a novel function for the *wee1* kinase. This function is presumably required for a normal cellular response to DNA-damaging agents. □

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- Hartwell, L. H. & Weinert, T. A. *Science* **246**, 629–634 (1989).
- Enoch, T. & Nurse, P. *Cell* **65**, 921–923 (1991).
- Enoch, T. & Nurse, P. *Cell* **60**, 665–673 (1990).
- Al-Khodairy, F. & Carr, A. M. *EMBO J.* **11**, 1343–1350 (1992).
- Rowley, R., Subramani, S. & Young, P. G. *EMBO J.* **11**, 1335–1342 (1992).
- Nurse, P. *Nature* **344**, 503–507 (1990).
- Featherstone, C. & Russell, P. *Nature* **349**, 808–811 (1991).
- Rowley, R., Hudson, J. & Young, P. G. *Nature* **356**, 353–355 (1992).
- Russell, P. & Nurse, P. *Cell* **49**, 559–567 (1987).
- Nurse, P. *Nature* **286**, 547–556 (1975).
- Mitchison, J. M. & Carter, B. L. A. *Meth. Cell Biol.* **11**, 201–219 (1975).
- Nurse, P. & Bisset, Y. *Nature* **292**, 558–560 (1981).
- Hagan, I. M. & Hyams, J. S. *J. Cell Sci.* **89**, 343–357 (1988).
- Hagan, I. M. thesis, Univ. London (1988).

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Direct visualization of botulinum neurotoxin-induced channels in phospholipid vesicles

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THE seven botulinum neurotoxin (NT) serotypes produced by strains of *Clostridium botulinum* inhibit neurotransmitter release from synaptic vesicles. Neurotoxin is synthesized as a roughly 150K single-chain protein. Proteolysis produces two fragments, the 50K L-chain and 100K H-chain, that remain linked by a disulphide bond. Intoxication involves membrane attachment by the C-terminal half of the H-chain, endocytotic/lysosomal internalization, vesicle channel formation mediated by the 50K N-terminal half of the H-chain at low pH, and finally blockade of synaptic vesicle fusion after the L-chain reaches the cytosol^{1–4}. We report here the visualization of the neurotoxin-membrane complex by electron cryomicroscopy and image processing. Three-dimensional reconstructions show the neurotoxin bound to the exterior of ganglioside/PC lipid vesicles and show channels entirely perforating the vesicle wall. Each channel appears to arise from the interaction of four neurotoxin molecules.

We induced ordered arrays of the NT by a modification of the phospholipid monolayer technique^{5,6} (Fig. 1 legend). Botulinum NT serotype B preferentially formed ordered arrays on the surfaces of vesicles and induced a shape change from their original spherical form to closed tubes (Fig. 1). Disordered bound NT often occurred near the ends of the tubes. To apply Bessel helical reconstruction techniques (Fig. 2), these images are first straightened⁷.

Figure 3 shows a section of a three-dimensional reconstruction. The interior can be displaced by negative stain⁸, so we assume that in these images the tubes enclose vitreous ice. The inner radius of the tube is 240 Å. We have chosen a threshold that results in a wall thickness which varies between 36 and 45 Å, except at the vesicle perforations (arrow). The inside of the wall is relatively smooth. We interpreted the protrusions on the outer surface to be botulinum NT molecules. They touch the vesicle wall, but much of the protein extends from the surface, forming loops. The maximum radius is about 405 Å. The channel (see below) is derived from the near-wall interaction of components of two loops. NT molecules also interact with each other a short distance above the wall surface, forming a bridge just above the channels (arrowhead).

Figure 4 shows the three-dimensional reconstruction. It represents the surface at which the density map exceeds a threshold. There are two views of the same reconstruction, one tilted about 25°, revealing the inside wall (Fig. 4a), and another almost exactly vertical (Fig. 4b). About 450 Å of the 1,080 Å helical repeat is shown here.

The most striking features of the inside wall are the channels that extend entirely through it. There are six helical rows of channels on the inside wall of the vesicle, two of which are visible on the far wall. The contour level was chosen to enclose a 40 Å phospholipid bilayer, thus we have an internal calibration for our surface representation. Using this cutoff the diameter of the

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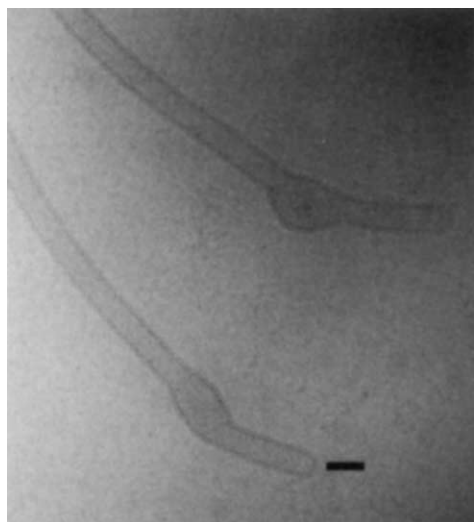


FIG. 1 An electron micrograph of frozen, hydrated botulinum NT vesicular tubes. In this print, protein is darker than ice for this unstained specimen. The micrograph was taken at $\times 30,000$ magnification. Scale bar, 1,000 Å. A solution of the NT (0.1 mg ml^{-1}) in 0.01 M citrate 0.005 M phosphate buffer was placed in a microtitre well at pH 5.6, under which conditions the NT forms channels in planar phospholipid bilayers¹³. This is close to the pH inside a developing lysosomal vesicle. A solution of ganglioside GT1_b (Supelco) and egg lecithin PC (Sigma) in a 1:20 weight ratio in chloroform was applied to the NT solution. Under our conditions, there is an excess of lipid, some of which forms vesicles in solution. Ordered arrays of protein formed on the lipid in a few hours at room temperature. A holey EM grid was applied to the surface to pick up about 3–5 μl suspension. After blotting and quick freezing in liquid ethane, we transferred the frozen grids to a Gatan cryoholder for the JEOL 1200 (100 kV) electron microscope and examined them at -150°C . The specimen had no carbon support film, and thus only protein, lipid and vitreous ice are imaged, as described previously¹⁴.

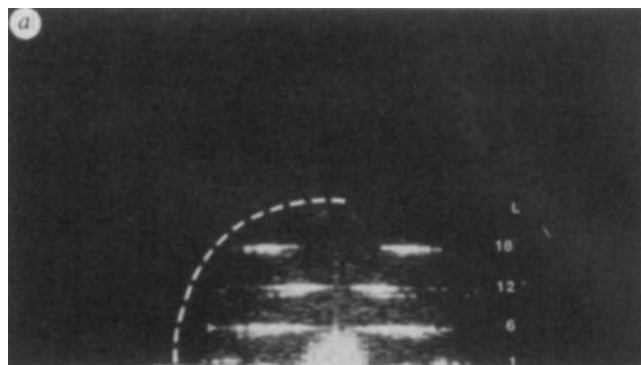
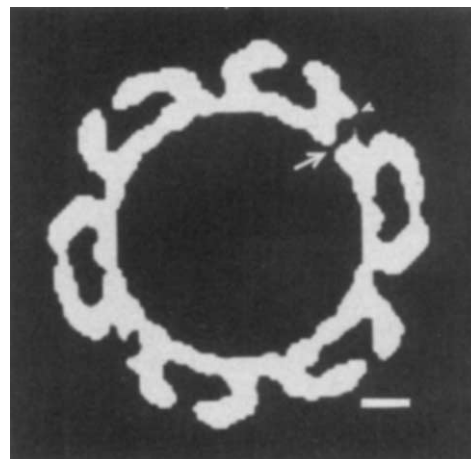


FIG. 2 Digitization was equivalent to 6.25 or 8.33 Å per pixel depending on magnification. The tubes were straightened by spline-fitting⁷. *a*, Summed Fourier transforms of 17 different straightened tubes from 7 micrographs. Only the top half is shown. This was used in determining the helical selection rule. The finest spacing between layer lines indicates a helical repeat of about 1,080 Å. Strong lines have a spacing of 1/6 this value (about 180 Å). The arrow indicates layer line 6. The dotted quarter-circle represents the furthest radial extent of the data used in the reconstruction (44 Å). The transform was indexed by a program that determines possible Bessel orders on a layer line, given the distance of the first peak from the meridian and the dimensions of the tube, then searches for selection rules that allow these orders. About five possible selection rules were found by this method. Constraints were applied by inspection of phases to distinguish odd from even Bessel orders and by observing that some tubes were curved out of the projection plane. The near and far sides thus appear in slightly different positions (not visible in the average in *a*), which allowed the relative assignment of sign. The ridges on the surface of the tubes were assigned a negative (left-handed) Bessel order from heavy-atom shadowing of the tube surface⁸. The only selection rule that obeyed all these constraints was 27 'units' in 28 turns. A 'unit' itself has sixfold character, as seen by the very strong J_{-6} (arrow), J_{-12} and J_{-18} Bessel orders that are prominent in *a*. In terms of this helical selection rule, there are 4 (see text) $\times 6 \times 27$ NT molecules per 28 turns. *b*, Plots of the layer lines used in the reconstruction. No correction was applied for the contrast transfer function with data of this resolution. Solid lines, amplitude; dotted lines, phase.

FIG. 3 Cross-section of the 3-D reconstruction from the image shown in Fig. 1. The reconstruction has a pseudo-6-fold appearance. The data set had a near side/far side Q factor¹⁵, measured as the ratio of vector sum to scalar sum, of > 0.81 . The phase residual between two independently processed data sets averaged 26° and the reconstructions had similar features. The reconstruction was done by standard helical methods¹⁶. Scale bar, 100 Å.



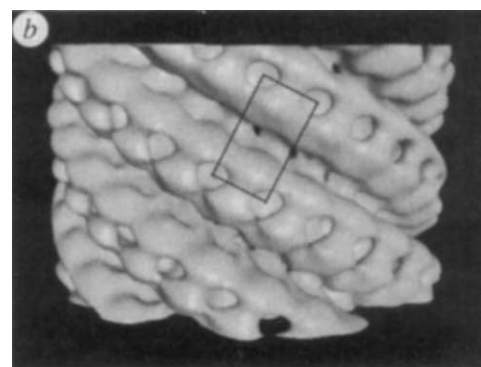
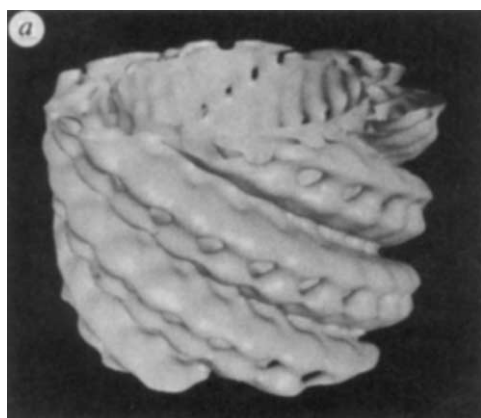
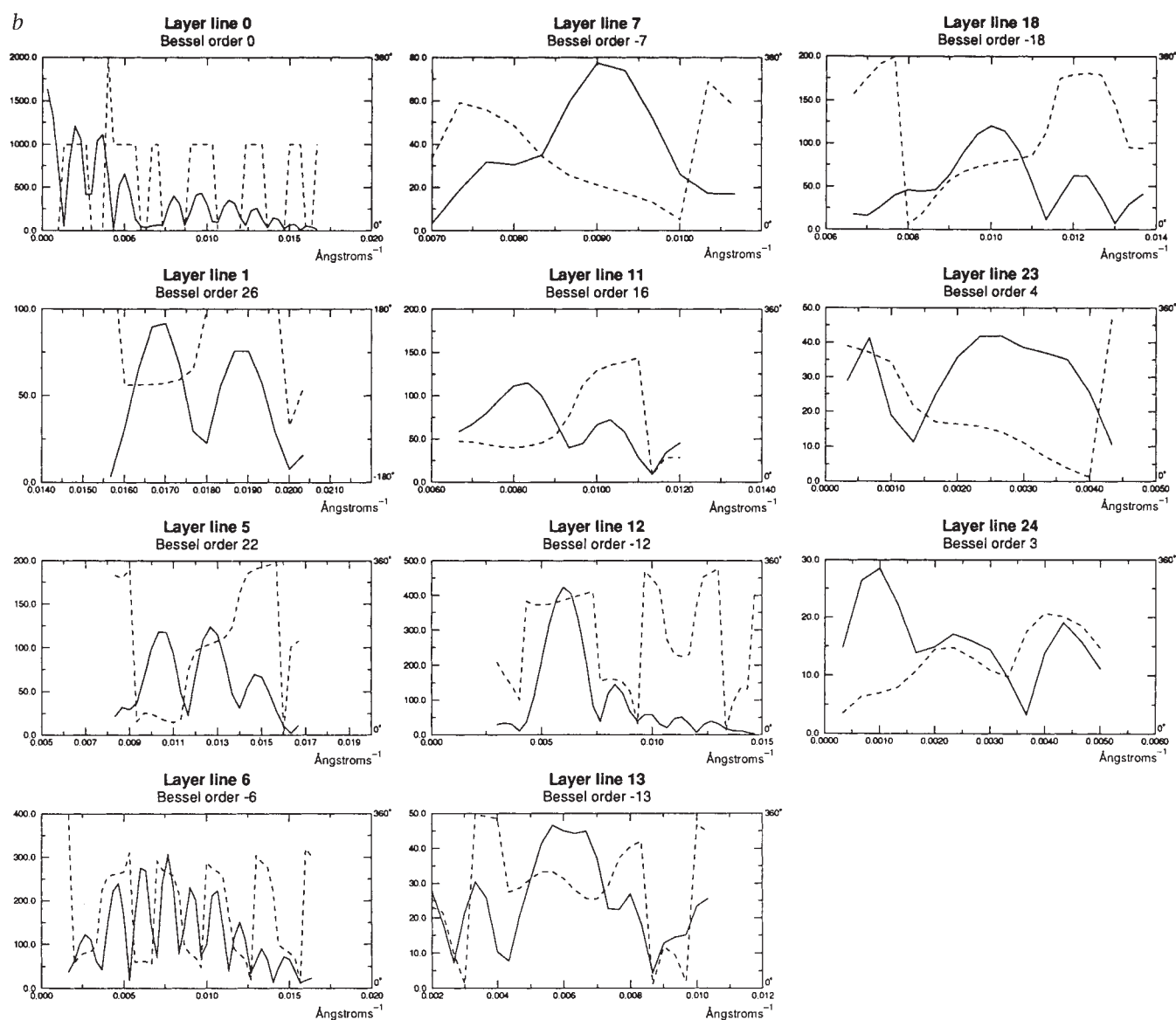


FIG. 4 A surface representation from the 3-D map from the image in Fig. 1. *a*, Tilted 25° from the vertical and *b*, with the helix axis vertical. The boundary of 2-fold related densities surrounding a single channel

is outlined in *b*. The contour level was chosen to give proper thickness to the lipid bilayer. Features are described fully in the text.

oval channel is about 14×25 Å and it varies somewhat among the 6 quasi-equivalent rows of channels. At the resolution of this reconstruction, the substructure or exact size of the channel cannot be determined. Studies of ion and molecular permeability through these channels⁹ indicate a size of about 20 Å. The outside of the tube is dominated by loops or ridges of protein that extend from the surface. The outside openings of the vesicle channels are at the base between the ridges.

Each channel arises from the interaction of protein densities from opposite sides of the channel. Using our contour cutoff, which correctly represented the phospholipid thickness, we calculated the total volume of the map exceeding the threshold. When we subtracted the volume occupied by a cylindrical annulus at 240–280 Å radius (the phospholipid), the remaining protein volume was 1.04×10^8 Å³ per helical repeat. Dividing by 27 units, and by 6 for the sixfold repeat of the channels, we arrive at about 640,000 Å³ of protein associated with each channel. This corresponds to 544K, about 4 times the M_r of each NT molecule. We thus interpret that the mass on either side of the channel is a dimer, so every channel is surrounded by four molecules. This indicates that a portion (10%) of the NT is embedded in the lipid, though our estimated error is of the same order (7%). It was suggested¹⁰ that for botulinum type C, aggregation into dimers or higher oligomers occurred during channel formation. Recently, 23 amino-acid tetanus toxin peptide has been shown to form channels in phospholipid bilayers¹¹. Molecular modelling produced a plausible 4-helix bundle structure whose α -helices have a centre-to-centre distance of 10–25 Å and surround a 5 Å pore. All NT studied so far contain at least one homologous amphipathic stretch in the N-terminal region of their heavy chain¹². Thus the channel may not be internal to each molecule; it could be formed cooperatively from several molecules after membrane insertion.

The bridge of density over the channel (arrowhead, Fig. 3) may be involved in the control of channel accessibility. Electrophysiological experiments¹⁰ implicated two alternating states, one of high and one of low conductance. The partial obstruction of the channel by the protein that we observe here could indeed be the time-averaged structural expression of this dynamic fluctuation. Alternatively, a channel with such an obstruction may influence the order of events that lead to channel formation. For example, *in vivo* the channel may form soon after the NT molecules bind to the cell surface, but in a partially blocked state (low conductance). Following endocytosis, the change in pH from near neutral to acidic could affect the uncovering of this channel, rather than its creation in the membrane. To decide between these mechanisms, this specimen will be studied under other conditions of external pH to see if other structural states can be observed. This is, to our knowledge, the first direct structural observation of a channel in a membrane formed from a soluble protein. □

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1. Simpson, L. L. (ed.) *Botulinum Neurotoxin and Tetanus Toxin* (Academic, San Diego, 1989).
2. DasGupta, B. R. *J. Physiol., Paris* **84**, 220–228 (1990).
3. Dolly, J. O. et al. *J. Physiol., Paris* **84**, 237–246 (1990).
4. Simpson, L. L. *A. Rev. Pharmacol. Toxicol.* **26**, 427–453 (1986).
5. Robinson, J. P., Schmid, M. F., Morgan, D. G. & Chiu, W. J. *molec. Biol.* **200**, 367–375 (1988).
6. Kornberg, R. D. & Darst, S. A. *Curr. Opin. Struct. Biol.* **1**, 632–646 (1991).
7. Chang, C. F. et al. *J. Ultrastruct. molec. Struct. Res.* **100**, 166–172 (1988).
8. Schmid, M. F., DasGupta, B. R. & Robinson, J. P. *Proc. XII Int. Congr. Electron Microscopy*, Seattle, 1990, 496–497 (San Francisco, 1990).
9. Hoch, D. H. & Finkelstein, A. *Ann. NY. Acad. Sci.* **456**, 33–35 (1985).
10. Donovan, J. J. & Middlebrook, J. L. *Biochemistry* **25**, 2872–2876 (1986).
11. Montali, M. S. et al. *FEBS Lett.* **313**, 12–18 (1992).
12. Binz, T. et al. *J. biol. Chem.* **265**, 9153–9158 (1990).
13. Hoch, D. H. et al. *Proc. natn. Acad. Sci. U.S.A.* **82**, 1692–1696 (1985).
14. Schmid, M. F. et al. *J. molec. Biol.* **221**, 711–725 (1991).
15. Grant, R. A. et al. *Biophys. J.* **49**, 251–258 (1986).
16. DeRosier, D. J. & Moore, P. B. *J. molec. Biol.* **52**, 355–369 (1970).

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Abbreviations, symbols, units and Greek letters should be identified the first time they are used. Acronyms should be avoided whenever possible and, if used, defined. Footnotes are not used in the text.

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